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13. ABSTRACT (Maximum 200 Words)

Hope for prevention of breast cancer arises form international breast cancer rate variation, which suggests potentially modifiable environmental determinants of the disease. We used immunocytochemical and Western blot methods to localize two membrane xenobiotic transporter, MRP1 and MRP2. MRP2 is located at the apical membrane of epithelial cells and excretes anionic hydrophobic substrates, such as 17 beta-estrogen glucuronide, antioxidants, and toxic molecules into lumen of the kidney, liver, and gut. In isolated and tissue cultured breast ductules, MRP2 is located predominantly I the apical region of luminal epithelial cells and thus might bioconcentrate toxic substrates into sealed, non-flushed lumens. In a comparison epithelium, Caco2 cells in culture, MRP2 is localized at the apical membrane only and is detected as bands at 60 and 190 kDa in blots, but in breast tissue cultures MRP2 is detected only at 60 kDa. MRP1 transports anionic hydrophobic molecules across the basolateral side of epithelial cells into the connective tissue. In isolated and tissue cultured breast ductules, MRP1 is localized in the cytoplasm, as is typical for numerous epithelial cells and is detected as a band at 190 kDa. Thus, overall transport of toxins to, and bioconcentration in, breast ductile lumens may be determined by a balance between these two transporters. We have also obtained a new critical piece of information: we have detected the mRNA for MRP2 in samples of breast reduction tissue.

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Breast cancer, xenobiotic transporter, carcinogen, antioxidant, bioprotection

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Overview of Final Report

This revised Final Report attempts to take into consideration the Editorial, Contractual, and Technical Issues raised in the Final Report Review:

1. **Observations should be described first in a section other than the Conclusions.** This has been completed in this version.
2. **Data are missing.** Much more data are included in the revised version.
3. **Data from years 1 and 2 should be summarized.** This has been completed
4. **Include Era of Hope abstract.** This abstract has been included.
5. **Explain or justify why the Final Report described the two tight junction papers.** I have offered a more complete explanation of why tight junctions are potentially important in breast epithelial cell health and cancer carcinogenesis. Also, the finding that genistein protects tight junctions from oxidative stress suggests that genistein in the diet might help hold mutated breast epithelial cells in place, and prevent their metastasis.
6. **All relevant materials should be listed as proper bibliographic citations.** This has been completed in the **Reportable Outcomes** section.
7. **Diminish attention paid to three unfounded proposals.** Only their titles are listed in the Revised Final Report.
8. **Respond to technical issues.** The Final Report reviewer offered some interesting suggestions concerning alternative routes of research in this area. Specifically, these comments included expressing human MRP2 cDNA in human cell lines, animal cells, or oocytes. This is an excellent idea, since it would help determine dietary molecules that could be transported by the MRP2 and thereby be concentrated in the lumen of human breast ductules. I would want to use this idea in an NIH grant if I could obtain the proper preliminary data for the grant.

TITLE: THE BIOCONCENTRATION HYPOTHESIS: POTENTIAL ROLE OF TRANSPORTERS IN BREAST DUCTULES

INTRODUCTION

We propose to examine the possibility that several membrane transporters, Pgp, MRP, and MOAT, could continually or episodically (under the influence of various steroid hormones or environmental factors) transport toxic molecules into the lumen of breast epithelia over long periods of time (years). This transport process could be very significant because the lumen of breast epithelia in nonlactating women would essentially be a stagnant lumenal pool: the connection to drain any of its secretory products is normally closed by a keratotic plug. Toxins transported into the lumen would be concentrated well above blood concentrations and would continue to diffuse from the lumen back to the cell, affording them time to damage or modify DNA. Using primary

tissue cultures of human breast epithelial tissue, we will determine which of these transporters is functionally present, what they can transport, what kind of concentration they can achieve in the lumen, and determine if their transport function is under hormonal control. To our knowledge, research on breast cancer has never been systematically approached with the hypothesis that a **number** of transporters could be present in ductal epithelia and consequently, bioconcentrate potentially carcinogenic molecules in the lumen. The detection of Pgp, MRP, and MOAT in normal breast epithelium has not been rigorously pursued by others because there was no rationale to do so in a highly concerted way (that is, with functional and biochemical studies), where this proposal provides this rationale. The effects of these transporters might be especially deleterious to the breast: during the first part of the ovulatory cycle estrogen might enhance expression, with the result that hydrophobic molecules are transported to the breast ductule lumen. Then later in the month progesterone concentration rises and could inhibit the upregulated Pgp, and the lining cells could not longer transport these hydrophobic molecules into the lumen. They would leak back into the lining cells.

BODY

TECHNICAL OBJECTIVES

Overview of progress on the seven tasks of the SOW:

There were several overriding considerations in completing these tasks:
1. Tissue availability was very limited, sometimes to a few surgeries per month, at best. When I talked to the surgeons as I was writing the grant proposal they stated that there were several surgeries per week. Clearly, this did not turn out to be the case.
2. Tissue was never given to me immediately after the operation, but at the end of that day or the morning of the next day, because of University Hospital rules. This was not fresh tissue and observations made in this tissue were not consistent. Even when we received the tissue, we needed to digest it with collagenase overnight to make isolated tubules. This tissue did not give consistent results. This problem led us to take this surgical tissue, digest it with collagenase overnight to make clumps of cells, and then to tissue culture these cells for the next few weeks as flat sheets that would potentially be useful for transport studies.

Task 1: Measuring function in cysts/cyst fluid

Because of the freshness issue, the lack of tissue, and inconsistent results obtained with these cultures, we devoted most of our time to a second preparation. Starting in the firswt year of this grant, we processed the cysts into tissue culture preparations as flat sheets of epithelial cells. These preparations are actually more ideal, since one has much better control over conditions for transport studies.

Task 2: Measuring transport in primary cultures.

We developed the techniques to successfully grow flat sheets of breast epithelial cells. These cultures exhibited a morphological pattern much like native breast epithelial cells. Thus these cells exhibited apical microvilli, tight junctions, and all the normal organelles. We localized MRP1 and MRP2 in these cultures with immunocytochemical methods. The localization of these transporters revealed that the MPR1 was present in the basolateral membrane, and MRP2, was in the apical cytoplasm and/or apical membrane, where it is located in several other epithelia (e.g., gut, liver, kidney). We also ran SDS-PAGE and attempted to identify MRP1 and MRP2 in the gels. MRP1 was there at the correct molecular weight of 190 kDa. Unfortunately, MRP2 always was present only at about 70 kDa. A companion gel run with membranes isolated from CaCo2 cells (an intestinal cancer cell line which also possesses MRP1 and MRP2) showed MRP1 and MRP2 of those cells was present at their correct molecular weights of 190 kDa. The fact that the molecular weight of MRP2 meant that is was not appropriate to perform transport studies on these tissues. We needed to try to get the MRP2 in the membrane at its correct molecular weight (190 kDa). We mounted some of these cultures in Ussing chambers to measure their electrical properties. These experiments showed that the breast epithelial cells were making tight junctions and exhibited a transepithelial electrical resistance of 200-1000 ohm-cm^2, which was excellent from the standpoint of epithelial integrity. The fact that the molecular weight of MRP2 was only 79 kDa meant that is was not appropriate to perform transport studies on these tissues.

Task 3: Measuring transport in commercial/primary cell lines

There was only one other commercial cell line available for us to try, HMEC (Clonetics). One of my collaborators, Dr. Tom Walle, who is excellent at growing all types of transporting epithelial cells had found that these cells do not form confluent sheets. In other words, they grow with scattered areas without any cells at all. There is no use in doing transport studies unless epithelial cells become confluent and form tight junctions. We did make numerous attempts to grow these cells in different culture conditions, but they remained non-confluent. Also, we attempted to detect MRP2 in these cells using our immunocytochemical methods. We finally succeeded in demonstrating the localization of this transporter. Also, these cells were making the tight junction protein, occluding, as detected by immunocytochemistry. However, there were still non-confluent patches in the cultures, so they are unusable for transport studies.

Task 4: Determine estrogen, progesterone effects on transport

We did not have a working model system of flat breast epithelial cells in which to measure transport, so these experiments were not done. In our first observations on transport into isolated ductules, we found that transport of Texas Red to the lumen was more common than when we did not expose the ductules to this hormone. Again, results were not consistent in those isolated ductules, and we focused most of our attention on the tissue culture models. From time to time we would use some of the precious isolated ductules for a transport study, attempting to see the effect of different inhibitors.

Task 5: Determine pesticide effects on transport

We did not have a working model to test the effects of pesticides on transport. We did not utilize the isolated ductules since they gave inconsistent results without pesticide.

Task 6: Immunocytochemical localization of transporters

As detailed above we localized MRP1 and MRP2 in these cultures with immunocytochemical methods. The localization of these transporters revealed that the MPR1 was present in the basolateral membrane, and MRP2, was in the apical cytoplasm and/or apical membrane, where it is located in several other epithelia (e.g., gut, liver, kidney).

Task 7: Measuring protein expression in primary cultures

As detailed above, we also ran SDS-PAGE and attempted to identify MRP1 and MRP2 in the gels. MRP1 was there at the correct molecular weight of 190 kDa. Unfortunately, MRP2 always was present only at about 70 kDa. A companion gel run with membranes isolated from CaCo2 cells (an intestinal cancer cell line which also possesses MRP1 and MRP2) showed MRP1 and MRP2 of those cells was present at their correct molecular weights of 190 kDa. The fact that the molecular weight of MRP2 meant that is was not appropriate to perform transport studies on these tissues. We needed to try to get the MRP2 in the membrane at its correct molecular weight (190 kDa). Because immunocytochemistry always carries the risk that another protein also binds to the antibody, we also detected the presence of the mRNA for MRP2 in our flat tissue cultures of isolated ductules.

Year One: 1998-1999 Highlights

Isolated ductule transport study with Texas Red (no transport detected). Trypan blue exposure showed no uptake, so cells had remained intact.

Start of tissue culture work: Isolated ductules placed into Transwells after overnight treatment with collagenase. Ductules lost their original morphology (were spreading out).

Isolated ductules transport study: ductules exposed to estrogen (10^{-8} M) and progesterone (10^{-7} M) for 24 hours showed Texas Red in their lumens. Control tissue without hormones showed no luminal fluorescence.

Further experimentation with different tissue culture media. Results showed that morphology of cultures was best if bovine pituitary extract was added to the tissue culture medium.

Isolated ductule transport study. Transport observed with and only with hormone treatment (estrogen plus progesterone). KCN did not inhibit transport, but CDNB, a nonfluorescent substrate of MRP2, did inhibit to some extent. No daunomycin (P glycoprotine substrate) transported to lumens.

Isolated ductule transport study. Small amount of transport observed with and only with hormone treatment (estrogen only in this case). CDNB inhibited transport.

SDS-PAGE. Lower molecular weight band (about 70 kDa) but no 190 kDa band detected in membranes extracted from tissue cultures.

SDS-PAGE. Lower molecular weight band (about 90 kDa) but no 190 kDa band detected in membranes extracted from tissue cultures. Observed 190 kDa band in positive control (Caco2 cells). Presence or absence of Epidermal Growth Factor and Bovine Pituitatry Extract made no difference.

First measurements of electrical properties of tissue cultures. In the first few culture attempts the electrical resistance was low (around 100 ohm-cm^2). However, later cultures the electrical resistance was around 200 ohm- cm^2.

New technician starts work.

SDS-PAGE. Lower molecular weight band (about 50 kDa) but no 190 kDa band detected in membranes extracted from tissue cultures. Observed 190 kDa band in positive control (Caco2 cells).

Further experimentation with different tissue culture media.

SDS-PAGE. Lower molecular weight band (about 60 kDa) but no 190 kDa band detected in membranes extracted from tissue cultures, no matter what tissue culture media used. Observed 190 kDa band in positive control (Caco2 cells).

Beginning of immunocytochemistry on isolated ductules and tissue cultures. Several months of work optimizing conditions (varying fixation and staining times, testing various commercial antibodies and antibodies gifted by another lab working in this field).

One research group has had success growing flounder proximal tubule cells in a special tissue culture preparation that induces greater expression of the Permeability glycoprotein. This technique involves the use of a contracting collagen gel, which leads to phenotypic changes in cell morphology (they become more columnar, as they are in the fish). We made several attempts at using this technique to help induce the expression of MRP2. We could not get the breast epithelial cells to stay attached to the collagen matrix, so this technique failed for us.

Year Two: 1999-2000 Highlights (this year saw a dearth in breast reduction surgeries)

We achieved success with immunocytochemistry after trying many variations of technique. We observed positive staining in the apical cytoplasm/apical membrane region with antibodies to MRP2, and in the basolateral region with MRP1.
To sum up our observations with Western blots on breast epithelial cells, we always (no matter how many variations of culture conditions or Western blot techniques) saw bands at 60-70 kDa for MRP2, whereas Caco-2 cells served as a control in all our Western blots, giving a signal at 190 kDA (and also a 60-70 kDa band like the breast epithelial cell preparations). Since we had repeatedly observed apical cytoplasm/apical membrane region staining in immunocytochemistry with breast epithelial cells (freshly isolated or in tissue culture) we wanted to double check Caco-2 cells with our immunocytochemical methods to see if there were a parallel with that technique. A colleague in a laboratory in the Department of Pharmacology here at MUSC was studying the transport of the dietary flavanoid quercetin in Caco-2 cells, so we collaborated with them. This allowed us to use this data not only for our own understanding of breast epithelial cells, but to get our name on one of their publications and **to place this grant number on that publication**. The paper was:
Walgren RA, Karnaky KJ Jr, Lindenmayer GE, Walle T.
Efflux of dietary flavonoid quercetin 4'-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. Pharmacol Exp Ther. 2000 Sep;294(3):830-6.

The study produced perhaps the best immunocytochemical micrograph of MPR2 localization published to that time. Interestingly we observed no immunocytochemical stain for MRP1in tissue sections, but we have always observed good staining of a band at 190 kDa in Western blots of Caco-2 cells.

More transport studies, which showed some transport if ductules incubated with estrogen.

Attempted immunocytochemistry on the only available commercial breast epithelial cell line, HMEC. Not successful, since cytoplasm and nuclei stained positively.

Because there were fewer breast reduction surgeries during this grant year we made a strong effort to obtain breast reduction tissue from a private hospital (a Breast Cancer Center) in the Charleston area. This took months of waiting on paperwork and phone calls. We finally were able to obtain one specimen, but they did not respond to any requests after that.

Another successful immunocytochemistry study of breast epithelial cells in culture.

Since this research project is focused on human breast epithelial cells during the decades prior to the development of breast cancer and the conditions that could lead to that development, we had not spend any time on breast cancer cells. But with the dearth of

normal breast reduction tissue, we decided to attempt to detect MRP2 in several breast cancer cell lines made available to use by labs at MUSC (MCF-7, MCF-10A, and MCF-neoT). MCF-10A, and MCF-neoT cells had cytoplasmic labeling with our immunocytochemical methods. MCF-7 cells had no cytoplasmic labeling. All of these cell lines showed nuclear labeling, which is difficult to explain.

Immunocytochemistry to determine if our breast epithelial cultures were expressing tight junction proteins. We observed a positive staining at the edges of cells with an antibody to ZO-1.

Continued attempts to detect tissue cultured breast epithelial cell MRP2 at 190 kDa in Western blots were not successful (lower bands only). These attempts included changing concentrations of Epidermal Growth Factor and Bovine Pituitary Extract.

Successful Texas Red transport study in ductules. As expected for Texas Red transport mediated by MRP2, inhibition should have been observed after exposure to CDNB and LTC4, and it was.

Further attempts to develop commercial breast epithelial cell line for transport studies. Performed immunocytochemistry to detect MRP2, and it was present in the apical region. However, these cells do not form a perfectly confluent monolayer, so they could not be used for transport studies.

Further immunocytochemistry on freshly frozen tissues. Positive immunocytochemical localization of MRP2 in breast ductules. As should have been observed, staining was blocked by peptide to protein sequence to which antibody was made.

Year Three: **2000-2001 Highlights**

In our previous immunocytochemical studies, which involved using frozen sections for maximal preservation of the transporter, MPR2 was localized in the apical region, and we could not be sure whether the transporter was in the apical membrane. We therefore attempted to detect MRP2 using more conventional fixation/paraffin embedding methods. These efforts did not offer improved results.

We began to measure electrical properties of our tissue cultured breast epithelial cells, after working out methods to grow the cells on special Transwell inserts. These Transwell inserts have a porous substrate and cells are grown on the top of the substrate with tissue culture media both on the top (mucosal side) and on the bottom (serosal side).

These cells became confluent and could be used for immunocytochemistry to localize MRP2, which was in the apical region. It took multiple attempts to work out immunocytochemical procedures for these inserts.

Cells took about 2 weeks to grow to confluency on these inserts. We observed transepithelial electrical resistances in the range of 300-1000 ohm-cm^2. Inserts with diameters of 6.5 mm gave better resistances than those with a diameter of 12 mm. We first measured these electrical properties with a device called the EVOM, which is used just for this purpose. The electrical property of resistance can be measured by placing the electrodes of this device directly into the well containing the insert. One electrode is positioned on the mucosal side and the other is one the serosal side. The only reliable data from this instrument is the transepithelial electrical resistance.

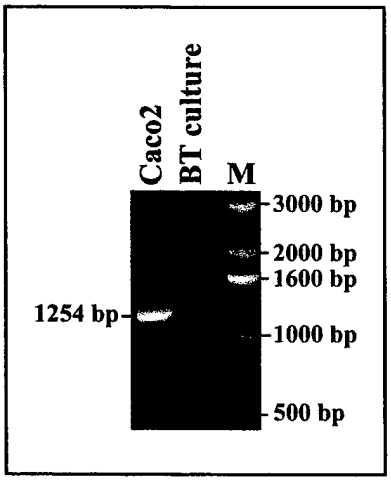
Western blots on these cultures showed that MRP1 was at the correct 190 kDa molecular weight, but that MRP2 stained only as the lower molecular weight bands 50-60 kDa, and not as 190 kDa bands.

We started mounting these inserts into Ussing chambers, which allow for the measurement of not only the transepithelial resistance, but also the transepithelial electrical potential and the current generated by any transport occurring in the epithelium. We observed resistances of approximately 1000 ohm- cm^2, and very small electrical potential differences and a very small short-circuit current (approximately 3 microamp/ cm^2. The small short-circuit current is much like that observed in Caco-2 cells that still exhibit a good deal of MRP2-mediated transport. These electrical resistances are greater than are observed in Caco-2 cultures.

Conducted addition transport studies in ductules, and observed Texas Red transport in several of the ductules. Inhibition studies with CDNB were successful.

Began attempting to detect mRNA for MRP2 in our breast epithelial cell cultures. This took a bit of time, but was successful.

MRP2 mRNA expression in Caco2 and breast epithelial cells (BT cultures) by reverse transcription PCR (RT-PCR)



Method: cDNA was generated with random examers and amplified using MRP2 specific primers (Schaub et al., *J. Am. Soc. Nephrol.* **10**:1159-69, 1999). The expected 1254 bp

MPR2 DNA fragment was detected in both Caco2 and BT cells cultured on plastic. **This is a very exciting discovery, since it means definitively that MRP2 is present in breast epithelial cells.** Alcorn J, Lu X, Moscow JA, McNamara PJ. (Transporter gene expression in lactating and nonlactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. J Pharmacol Exp Ther 2002 Nov;303(2):487-96) reported MRP2 (and 18 other transporters) in isolated breast epithelial cells using the technique of RT-PCR.

Seven months into this third year my technician quit. It took several months to do the paperwork and interview for another technician, and I finally hired a student just out of college to start on June 1. However, within two months, she quit to go to graduate school. By now, the grant had one month left, and very little money.

The no-cost extension year, 2001-2002

During the last year of a no-cost extension I did not have enough funds to employ a technician, and no new laboratory data was generated. There were only a small amount of dollars left from the third year, and no technician would work for just a few months. Even though there was no data collected directly related to the grant, the no-cost extension allowed me to accomplish the following important grant-related items during **that year** and **start initiatives** to continue with this important research started by the Army Breast Cancer Program years ago. This list of 12 items demonstrates that we have made the best progress we could with the start of this project with Army Breast Cancer research funds. It also shows that I am committed to continuing this project as long as I can find a few dollars to purchase supplies.

1. Publication of a paper on genistein, a molecule known to be important in breast cancer prevention in rats.
[THIS PAPER ACKNOWLEDGED THE ARMY BREAST CANCER GRANT]

This research was not mentioned in the SOW for this grant, but collaborating with the investigator already working on this molecule added insight into my studies of transporters in breast cancer carcinogensis. **During the grant period this molecule was reported to be a substrate of MRP2, the tranporter I am focusing on.** This finding made it an especially important molecule and one worth studying. It is well known that breast cancer rates are lower in Eastern societies. It has been speculated that genistein in the soy so widely consumed in Eastern societies might play a role in this prevention. It is also known that administering genistein to prepubertal rats can prevent breast cancers by 80%. I was fortunate to be able to collaborate with a successful researcher here at MUSC on a project to determine the role of genistein in preventing oxidative damage to tight junctions in an intestinal cell line. We were able to publish a paper on this topic. The methods and insights in this paper *can be used in future studies* to determine the possible protective effect of genistein on breast epithelial cells in future experiments. Since MPR2 transports genistein, this transporter may determine the concentration of this molecule in and around breast epithelial cells. **It is important to note that genistein might hold mutated breast epitheial cells together tighter, and not let them escape**

(metastasize) from their original location. I thus started an important potential collaboration with Dr. R. K. Rao, who is interested in pursuing this line of research in breast epithelial cells the future. I wanted to take advantage of working with this colleague. **The next breast cancer grant I would submit would definitely include experiments on the transport of genistein by breast epithelial cells, based on the report that it is transported by MRP2 and that it could play a significant role in determining how well breast epithelial cells remained attached.**

Rao R, Basuroy S, Rao VU, Karnaky Jr KJ, and Gupta A. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. **Biochem J.** 2002 Dec 1;368(Pt 2):471-81.

2. Manuscript on MRP2 transport in shark rectal gland secretory tubules. We hope to apply the methodology utilized in this publication to the study of tissue cultured breast epithelial cells when they are cultured in the form of ductules. This study showed that MRP2 is regulated by endothelin, a molecule that would be expected to be present in inflammatory tissue. This paper is in a handful of first papers on hormonal regulation of MRP2, so this is an important thrust for future breast cancer research. If noxious agents irritate breast epithelial cells, then endothelin might be secreted into the inflamed area, thus regulating the transport activity of MPR2 in breast epithelial cells. **The next breast cancer grant I would submit would definitely include experiments on the role of endothelin in regulating transport of genistein and other MRP2 substrates by breast epithelial cells. I would put my experience on endothelin regulation of MRP2 to use in that grant proposal. This work with the shark rectal gland has laid the groundwork for future experiments on human breast epithelial cells.**
Research funded by a grant other than the Army Breast Cancer grant.
[THIS PAPER DID *NOT* ACKNOWLEDGE THE ARMY BREAST CANCER GRANT]

David S. Miller, Rosalinde Masereeuw and Karl J. Karnaky, Jr. Regulation of mrp2-mediated transport in shark rectal salt gland tubules. **American Journal of Physiology.** 282:R774-781, 2002.

3. I also want to list a review article on tight junctions in bony fish to show my expertise on the area of tight junctions. As noted above, genistein in the diet may help protect tight junctions in breast epithelial cells. This area of research will be pursued in future grants if at all possible. MRP2 in breast epithelial cells could transport genistein to the lumen of breast ductules, and help protect breast epithelial cell tight junctions against oxidative stress. I believe my expertise in tight junctions of epithelial cells will apply to breast epithelial cell health and cancer carcinogenesis. **The next breast cancer grant I would submit would definitely include experiments on the effect of genistein on tight junctions in breast epithelial cells. I would put my experience on tight junctions to use in that grant proposal.** Research funded by a grant other than the Army Breast Cancer grant.
[THIS PAPER DID *NOT* ACKNOWLEDGE THE ARMY BREAST CANCER GRANT]

Karnaky, K. J., Jr. Teleost chloride cell tight junctions: Environmental salinity and dynamic structural changes. In: **Tight Junctions (2nd edition)** (Ed. by M. Cereijido and J. Anderson) CRC Press, Boca Raton, pp. 445-458, 2001.

4. Through the generosity of the Army Breast Cancer program I was invited to attend the recent Army Breast Cancer Program Era of Hope meeting in Florida in September, and present my data. I received very positive feedback and encouragement, and some excellent ideas for future experiments in talking to numerous experts in the field of breast cancer. The poster was entitled: "BREAST EPITHELIAL CELL MEMBRANE TRANSPORTERS MAY DETERMINE EXPOSURE OF CELLS TO ANTIOXIDANTS AND CARCINOGENS" by **K. J. Karnaky, Jr., M. Sedmerova, W. Pendarvis, G. Re, and D. Hazen-Martin [ABSTRACT INCLUDED]**

5. During the year, 2001, I applied for external funding from three different agencies (grant titles listed below). Unfortunately, none of these were funded. In each case the reviewers wanted me to present preliminary data that the tissue cultured breast epithelial cells **would transport molecules relevant to breast cancer.** At the present time, and with our current methods, we have found that the MRP2 transporter protein degrades to a lower molecular weight. Because of objections in the Review of the original final report, I have deleted the Specific Aims:

A. **Funding agency: Susan B. Komen Breast Cancer Foundation**
Grant title: "A novel bioassay for genotoxic and protective effects of dietary and endogenous molecules"

B. **Funding agency: American Institute for Cancer Research**
Grant Title: "MRP Transporter Roles in Dietary Exposure of Breast Epithelial Cells"

C. **Funding agency: National Institutes of Health**
Grant Title: "MRP transport processes in breast epithelial cells"

6. This year extension year has given me the chance to apply for a small grant from my institution to try to gather this necessary preliminary data. The first application was rejected (application date, Nov, 30, 2001). However, I rewrote the proposal and submitted it on March 1, 2002). **This grant was funded, with funds starting in the Fall of 2002.** I am still using these funds to continue this research (March, 2004).

7. I was in the process of moving my laboratory to another building in the medical complex at the time of the original submission of the Final Report. I have made the move into that laboratory and unpacked as many boxes as the small space will allow.

8. I conducted research again the summer of 2002 at the Mt. Desert Island Biological Laboratory in Salisbury Cove, ME. An international group of experts does research here each summer on drug transporter proteins. I discovered that a researcher trying to work with the MRP2 transporter in human kidney experienced some of the same problems we have working with this transporter protein in breast epithelial cells.

9. We know we are on the right track in our pursuit of the function of MRP2 in breast epithelial cells, because of two new reports of the presence of MRP2 in breast cells.

A. Sandusky GE, Mintze KS, Pratt SE, Dantzig AH. Expression of multidrug resistance-associated protein 2 (MRP2) in normal human tissues and carcinomas using tissue microarrays. Histopathology 2002 Jul;41(1):65-74.

Sandusky et al., found MRP2 in 25 of 49 breast carcinomas, using immunocytochemical methods.

B. Alcorn J, Lu X, Moscow JA, McNamara PJ.: Transporter gene expression in lactating and nonlactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. J Pharmacol Exp Ther 2002 Nov;303(2):487-96

This group reported MRP2 (and 18 other transporters) in isolated breast epithelial cells using the technique of RT-PCR.

10. I (with Tom Walle) again organized a meeting (our fourth) on transporters: Metabolism and Transport of Xenobiotic and Endogenous Molecules "SYMPOSIUM" FRIDAY, MAY 10, 2002 8:50 AM-4:10 PM

11. This local institutional grant has allowed me to continue to study the phenomenon of transporters in breast epithelial cells. The Army Breast cancer grant I am now addressing in this Final Report allowed me to make enough progress that I could get the institutional grant funded. Briefly, I was fortunate to obtain immortalized breast epithelial cells that grow quickly in monolayer culture, form tight junctions (as shown by the presence of two junctional proteins, Z0-1 and occluding), and, in preliminary studies, appears to exhibit a net excretion of Texas Red, a substrate of MRP2. Unfortunately, these cultured cells also exhibit the lower molecular weight bands in SDS-PAGE, exactly like the cultures we made from breast reduction operations. Using information I have obtained attending the Army Breast Cancer meeting and the Experimental Biology meeting (which I attend each year), I will be undertaking new approaches to coaxing the immortalized breast epithelial cells into expressing MRP2 at the correct molecular weight. The strategy will utilize Matrigel as the substrate upon which to grow cells. Mina Bissell has shown that by growing breast epithelial cells on this substrate the cells secrete a normal protein, casein. If they are not grown on the Matrigel, they do not secrete casein. I will be working with the hypothesis that so far I have grown the cells on substrates that lack some special signal that tells MRP2 to remain at 190 kDa and to get into the apical cell membrane at that molecular weight.

12. I have published a paper on the role of breast epithelial cell transporters in breast health and carcer carcinogenesis. This review lays out my major ideas on the transporters and breast cancer and will help spread the word on this view of breast epithelial cell transporters. So far, there are very few publications relating transporters to breast cancer except for their possible role in multidrug resistance. My idea is that the transporters actually transport either salutary or harmful molecule to the lumen of these epithelial

cells of the breast ductule, where they could cause harm or benefit. **[THIS PAPER ACKNOWLEDGED THE ARMY BREAST CANCER GRANT]**

Karnaky, K. J., Jr., Hazen-Martin, D., and Miller, D. S. The xenobiotic transporter, MRP2, in epithelia from insects, sharks, and the human breast: implications for health and disease. J Exp Zoolog Part A Comp Exp Biol. 2003 Nov 1;300(1):91-7.

In summary, this no-cost extension of the grant allowed me to secure a modest level of funding from my home institution to continue the work on the MRP2 transporter in breast epithelial cells, and to continue to do research, attend meetings and journal clubs, and teach in this field. Finally, two papers acknowledging the Army Breast Cancer grant were published in 2002-2003 and one in 2000.

KEY RESEARCH ACCOMPLISHMENTS

- **Established that human breast epithelial cells, freshly isolated, and grown in culture, possess MRP2 in their apical cytoplasm or apical membrane.**
- **Developed methods to study these cells by immunocytochemistry, tissue culture, and molecular biology methods.**
- **MRP2 mRNA isolated; RT-PCR revealed a product at 750 bp. This means the transporter is present in breast tissue, with the most likely cell type being the breast epithelial cell.**
- **Uncovered, unfortunately, the fact that under all conditions we have used, the transporter is present in our cultures at the wrong molecular weight.**
- **Published three papers on the topics of: 1)MRP2 transport in breast epithelial cells (review article); 2) MRP2 localization in Caco-2 cells, the major cell line we use for positive controls in our studies of breast epithelial cells; 3) actions of genistein, a molecule that is present at high levels in populations in which breast cancer incidents are low, and it present in breast nipple aspirate fluid and breast milk in women who eat soy products.**
- **Used the above information to obtain a small grant from my school to further study this interesting biological problem.**
- **Have a better understanding of potential future research directions, since we are now aware of so many things NOT to try.**

REPORTABLE OUTCOMES

Results were reported (poster) at the Experimental Biology meeting in Orlando, FL, March/April, 2001. (Published abstract in APPENDIX)

Results were reported (invited poster) at the Environmental Mutagen Society meeting on Breast Cancer, held in Raleigh, NC, Sept. 24-27, 2001. Our poster presentation on breast epithelial cell transporters stimulated a tremendous amount of interest, and, in addition, led to an attractive invitation to write a review on the topic for a special issue of the Society's journal to record that meeting (not able to complete this review because of heavy teaching commitment at that time).

Results were reported with a talk at a MINISYMPOSIUM: Epithelial Metabolism and Transport of Xenobiotic and Endogenous Molecules, May 11, 2001, at the Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC (organized by Karl J. Karnaky, Jr. and Tom Walle).

Abstract for the American Society of Gastroenterology, Meetings. R.K. Rao and Karl J. Karnaky, Tyrosine kinase-dependent dissociation of occludin-zo-1 and e-cadherin-β-catenin complexes from the cytoskeleton during oxidative stress-induced disruption of tight junction.

Rao R, Basuroy S, Rao VU, Karnaky Jr KJ, and Gupta A. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. Biochem J. 2002 Dec 1;368(Pt 2):471-81. (Included in Appendix)

Walgren RA, Karnaky KJ Jr, Lindenmayer GE, Walle T. Efflux of dietary flavonoid quercetin 4'-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. Pharmacol Exp Ther. 2000 Sep;294(3):830-6. (Included in Appendix)

Karnaky, K. J., Jr., Hazen-Martin, D., and Miller, D. S. The xenobiotic transporter, MRP2, in epithelia from insects, sharks, and the human breast: implications for health and disease. J Exp Zoolog Part A Comp Exp Biol. 2003 Nov 1;300(1):91-7. (Included in Appendix)

CONCLUSIONS

Together, breast ductules and lobular epithelial cells are the major site of breast cancer carcinogenesis. Breast ductules can be isolated as tubes with sealed lumens, thus simulating normal, non-lactating epithelial breast tissue. We utilized confocal fluorescence microscopy to detect the potential transport of fluorescent substrates into the lumen of these isolated ductules.. There is little transport in untreated tubules, but about half of the tubules exposed to 10^{-8} M estrogen for 12 hours exhibit secretion and luminal concentration of sulforhodamine 101, a substrate of the multidrug resistance protein transporter, MRP2. Transport to the lumen is often inhibited by a second, specific, but non-fluorescent, substrate of the MRP2, a compound called CDMB. MRP2 is localized to the apical membrane region of lining breast ductal epithelial cells by

immunocytochemical methods. MRP2 normally excretes hydrophobic substrates, such as 17 β-estrogen glucuronide, antioxidants, and certain toxic molecules into fluid-flushed lumens of the kidney, liver, and intestines. In the non-lactating breast ductules, MRP2 would bioconcentrate toxic substrates into sealed, non-flushed lumens. We also have exciting new immunocytochemical and Western blot evidence for the presence of MRP1, which normally transports anionic hydrophobic molecules from the basolateral side of epithelial cells. Overall transport to the lumen may be determined by a balance between the level of activity of these two transporters in breast ductules and possibly the manner in which these transporters are regulated by endogenous and exogenous hormones and hormone mimics. Isolated breast ductules offer a useful approach to this bioconcentration phenomenon and its regulation by natural and environmental estrogen hormone mimics and its potential role in breast cancer carcinogenesis.

So what

The concept that breast cancer may be preventable derives from data on the international variation in breast cancer rates. This variation may be an indicator that there are potentially modifiable environmental and lifestyle determinants of breast cancer. Numerous migration studies reinforce this premise. It has been found that Japanese immigrants to the United States acquire much of the breast cancer risk of the host country within two generations (Kliewer and Smith, 1995). The **BIOCONCENTRATION** idea under exploration in this grant would help relate specific carcinogenic food molecule that are substrates of MRP1 and MRP2. MRP2 could potentially transport and concentrate these in breast ductule lumens. Since the transported substrates in the lumen might be hydrophobic, they could cross the apical membrane and damage epithelial cell DNA. On the other hand, MRP1 might provide **PROTECTION** from these same carcinogenic food molecules if it were actively transporting them out of the cell and back to the connective tissue. The interplay between the relative activities of these two transporters could determine the environment surrounding ductule epithelial cell DNA, where most breast cancer develops. Much further work will be required to understand the roles of these two transporters in breast cancer carcinogenesis. Since MRP1 and MRP2 have specific substrates, researchers should be able to search for specific food molecules involved in transport processes involving bioconcentration and protection.

Final note for this Revised Final Report: This Army Breast Cancer Program grant has allowed our research group to progress far beyond the initial, simple idea that certain epithelial transporters could be involved in breast cancer. This research has come to the point that we and others have shown that such transporters are present in breast epithelial cells and have allowed our group to develop methods to tissue culture these breast epithelial cells so that they could be studied in special plastic chambers (Ussing Chambers). Unfortunately, at the present time the MPR2 transporter degrades to a lower molecular weight than normal. Our current modest grant will focus on experiments to

atttempt to induce the transporter to the proper molecular weight so that this model system can be used to understand transport phenomena in breast epithelial cells. To our knowledge, no other laboratory is pursuing this pathway. We could never have explored this pathway were it not for the Army Breast Cancer program.

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Schaub, T. P., Kartenbeck, J., Konig, J., Spring, H., Dorsam, J., Staehler, G., Storkel, S., Thon, W. F., Keppler, D. J. Expression of the MRP2 gene-encoded conjugate export pump in human kidney proximal tubules and in renal cell carcinoma. Am. Soc. Nephrol. **10**:1159-69, 1999

APPENDICES (Publications acknowledging this grant):

The following publications are attached:

Rao R, Basuroy S, Rao VU, Karnaky Jr KJ, and Gupta A. Tyrosine phosphorylation and dissociation of occludin-ZO-1 and E-cadherin-beta-catenin complexes from the cytoskeleton by oxidative stress. **Biochem J.** 2002 Dec 1;368(Pt 2):471-81.

Walgren RA, Karnaky KJ Jr, Lindenmayer GE, Walle T. Efflux of dietary flavonoid quercetin 4'-beta-glucoside across human intestinal Caco-2 cell monolayers by apical multidrug resistance-associated protein-2. Pharmacol Exp Ther. 2000 Sep;294(3):830-6.

Karnaky, K. J., Jr., Hazen-Martin, D., and Miller, D. S. The xenobiotic transporter, MRP2, in epithelia from insects, sharks, and the human breast: implications for health and disease. J Exp Zoolog Part A Comp Exp Biol. 2003 Nov 1;300(1):91-7.

The following published abstract is attached:

K. J. Karnaky, Jr., M. Sedmerova, W. Pendarvis, and D. Hazen-Martin. Immunocytochemical localization of MRP1 and MPR2 in human breast epithelial cells. FASEB J. 15, A836, 2001.

The following unpublished abstract from the Fall, 2002, Army Breast Cancer meeting is attached:

K. J. Karnaky, Jr., M. Sedmerova, W. Pendarvis, G. Re, and D. Hazen-Martin. Breast epithelial cell membrane transporters may determine exposure of cells to antioxidants and carcinogens.

Published in FASEB J. 15, A836, 2001.

Immunocytochemical localization of MRP1 and MPR2 in human breast epithelial cells. K. J. Karnaky, Jr., M. Sedmerova, W. Pendarvis, and D. Hazen-Martin, Depts. of Cell Biol. and Anat. and Path. and Lab. Med., and the Marine Biomed. and Environ. Sci. Prog., Med. Univ. of SC, Charleston, SC, 29425.

Hope for prevention of breast cancer arises from international breast cancer rate variation, which suggests potentially modifiable environmental determinants of the disease. We used immunocytochemical and Western blot methods to localize two membrane xenobiotic transporters, MRP1 and MRP2. MRP2 is located at the apical membrane of epithelial cells and excretes anionic hydrophobic substrates, such as 17 beta-estrogen glucuronide, antioxidants, and toxic molecules into lumens of the kidney, liver, and gut. In isolated and tissue cultured breast ductules, MRP2 is located predominantly in the apical region of luminal epithelial cells and thus might bioconcentrate toxic substrates into sealed, non-flushed lumens. In a comparison epithelium, Caco2 cells in culture, MRP2 is localized at the apical membrane only and is detected as bands at 60 and 190 kDa in blots, but in breast tissue cultures MRP2 is detected only at 60 kDa. MRP1 transports anionic hydrophobic molecules across the basolateral side of epithelial cells into the connective tissue. In isolated and tissue cultured breast ductules, MRP1 is localized in the cytoplasm, as is typical for numerous epithelial cells and is detected as a band at 190 kDa. Thus, overall transport of toxins to, and bioconcentration in, breast ductule lumens may be determined by a balance between these two transporters. Supported by DOD grant DAMD17-98-8125.

Fall 2002 Army Breast Cancer Meeting

BREAST EPITHELIAL CELL MEMBRANE TRANSPORTERS MAY DETERMINE EXPOSURE OF CELLS TO ANTIOXIDANTS AND CARCINOGENS

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Hope for prevention of breast cancer arises from international breast cancer rate variation, which suggests potentially modifiable environmental determinants of the disease. We used immunocytochemical and Western blot methods to localize two membrane xenobiotic transporters, MRP1 and MRP2. MRP2 is located at the apical membrane of epithelial cells and excretes anionic hydrophobic substrates, such as 17 beta-estrogen glucuronide, antioxidants, and toxic molecules into lumens of the kidney, liver, and gut. In isolated and tissue cultured breast ductules, MRP2 is located predominantly in the apical region of luminal epithelial cells and thus might bioconcentrate toxic substrates into sealed, non-flushed lumens. In a comparison epithelium, Caco2 cells in culture, MRP2 is localized at the apical membrane only and is detected as bands at 60 and 190 kDa in blots, but in breast tissue cultures MRP2 is detected only at 60 kDa. In other epithelia, MRP1 transports anionic hydrophobic molecules across the basolateral side of epithelial cells into the connective tissue. In isolated and tissue cultured breast ductules, MRP1 is localized in the cytoplasm, as is typical for numerous epithelial cells and is detected as a band at 190 kDa. Thus, overall transport of toxins to, and bioconcentration in, breast ductule lumens may be determined by a balance between these two transporters. Human nipple aspirate fluid and breast milk contain a number of MRP2 substrates, suggesting that breast epithelial cells have both the metabolic pathways (P450 and conjugating enzymes) and transporters, such as MRP2, to metabolize certain hydrophobic molecules which enter the basal side of the cell from connective tissue.

Tyrosine phosphorylation and dissociation of occludin–ZO-1 and E-cadherin–β-catenin complexes from the cytoskeleton by oxidative stress

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The oxidative-stress-induced alteration in paracellular junctional complexes was analysed in Caco-2 cell monolayer. Oxidative stress induced a rapid increase in tyrosine phosphorylation of occludin, zonula occludens (ZO)-1, E-cadherin and β-catenin. An oxidative-stress-induced decrease in transepithelial electrical resistance was associated with a redistribution of occludin–ZO-1 and E-cadherin–β-catenin complexes from the intercellular junctions. Genistein, a tyrosine kinase inhibitor, prevented the oxidative-stress-induced decrease in resistance and redistribution of protein complexes. Occludin, ZO-1, E-cadherin and β-catenin in the Triton-insoluble cytoskeletal fraction were reduced by oxidative stress, which was prevented by genistein. Oxidative stress also reduced the co-immunoprecipitation of ZO-1 with occludin, which was prevented by genistein. Co-immunoprecipitation of β-catenin with E-cadherin was unaffected by oxidative stress or genistein. ZO-1, E-cadherin and β-catenin in the plasma membrane or membrane-cytoskeleton were either slightly reduced or unaffected by oxidative stress or genistein. These results show that oxidative stress induces tyrosine phosphorylation and cellular redistribution of occludin–ZO-1 and E-cadherin–β-catenin complexes by a tyrosine-kinase-dependent mechanism.

Key words: cell–cell adhesion, epithelium, paracellular permeability, tight junction, tyrosine kinase.

INTRODUCTION

The tight junctions (TJs) and the adherens junctions (AJs) form the barrier to the diffusion of macromolecules across the epithelium in different tissues. Whereas TJs form a physical barrier to the diffusion of macromolecules through the paracellular space, AJs may indirectly regulate the structure and function of TJs. Studies during the past decade have identified a number of specific proteins localized at the TJ and AJ. Occludin [1], claudins [2] and junction adhesion molecule [3] are the transmembrane proteins currently known to be localized at the TJ. Zona occludens (ZO)-1, ZO-2 and ZO-3 are the major TJ-plaque proteins, which bind to intracellular domain of occludin [4,5]. The interaction between occludin and ZO-1 plays a crucial role in maintaining the structure of TJ and epithelial barrier function [6]. On the other hand, E-cadherin (the transmembrane protein) and catenins (the AJ-plaque proteins) are the major proteins localized at the AJ [1]. Binding of β-catenin to E-cadherin plays an important role in the maintenance of the structure of AJs [7–9]. Additionally, a variety of intracellular signalling molecules have been localized at the TJ and AJ [1], which suggested the possible role of signalling pathways in the regulation of the structure and function of TJs and AJs. A significant body of evidence indicates that TJs and paracellular permeability are regulated by signalling molecules, such as intracellular calcium [10], cyclic AMP [11], rho GTPases [12,13], G-proteins [14,15] and protein kinases [16–26].

We have previously shown that oxidative stress increases the paracellular permeability in Caco-2 cell monolayers without affecting the cell viability [16,17]. This effect of oxidative stress was caused by glutathione oxidation and inhibition of protein tyrosine phosphatases [18]. Oxidative-stress-induced paracellular permeability was prevented by tyrosine kinase inhibitors, and was associated with tyrosine phosphorylation of a wide spectrum of proteins [16]. The specific role of protein tyrosine phosphorylation in the mechanism of the oxidative-stress-induced increase in paracellular permeability is not clear. However, evidence suggests that tyrosine phosphorylation of junctional proteins may play a role in regulation of cell–cell adhesion [20,27,28]. Therefore oxidative-stress-induced tyrosine phosphorylation may disrupt the protein complexes of TJs and AJs.

In the present study, we determined the effect of oxidative stress on occludin–ZO-1 and E-cadherin–β-catenin complexes, and analysed the tyrosine phosphorylation of these paracellular junctional proteins. This study shows that oxidative stress: (i) induces tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin, (ii) dissociates the occludin–ZO-1 and E-cadherin–β-catenin complexes from the intercellular junctions by a tyrosine kinase-dependent mechanism, (iii) dissociates the occludin–ZO-1 complex, but not the E-cadherin–β-catenin complex, (iv) dissociates the occludin–ZO-1 and E-cadherin–β-catenin complexes from the cytoskeletal fraction by a tyrosine-kinase-dependent mechanism, and (v) induces tyrosine phosphorylation of cytoskeleton-associated ZO-1 and β-catenin.

EXPERIMENTAL

Chemicals

Cell culture media and related reagents were purchased from Gibco-BRL (Grand Island, NY, U.S.A.). Xanthine oxidase, xanthine, streptavidin agarose and protein-A Sepharose were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade purchased either from Sigma Chemical Company or Fisher Scientific (Tustin, CA, U.S.A.).

Abbreviations used: AJ, adherens junction; HRP, horseradish peroxidase; TER, transepithelial electrical resistance; TJ, tight junction; XO+X, xanthine oxidase+xanthine; ZO, zonula occludens.

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Table 1 Effect of genistein on XO + X-induced paracellular permeability

The basal TER of monolayers varied from 400 to 500 $\Omega \cdot cm^2$. Values are means ± S.E.M. ($n = 4$). Results that are significantly ($P < 0.05$) different from values for control (*) or 3 h XO + X monolayers (†) are indicated.

Property	0 h	1 h	3 h	3 h + genistein
Basal TER (%)	103 ± 5	72 ± 4*	30 ± 6*	89 ± 7†
Mannitol flux (%/h/cm²)	0.21 ± 0.02	0.25 ± 0.02	0.85 ± 0.06*	0.23 ± 0.02†

Antibodies

Mouse monoclonal anti-occludin, and rabbit polyclonal anti-occludin and anti-(ZO-1) antibodies were from Zymed Laboratories Inc. (South San Francisco, CA, U.S.A.). Mouse monoclonal anti-(E-cadherin) and anti-(β-catenin), biotin-conjugated anti-phosphotyrosine and horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibodies were from Transduction Laboratories (Lexington, KY, U.S.A.). FITC-conjugated anti-phosphotyrosine, Cy3-conjugated goat anti-rabbit IgG and FITC-conjugated anti-mouse IgG were from Sigma Chemical Co., and rabbit polyclonal anti-(E-cadherin) and anti-(β-catenin) antibodies were from Chemicon International Inc. (Temecula, CA, U.S.A.).

Cell culture

Caco-2 cells, purchased from the American Type Culture Collection (ATCC; Rockville, MD, U.S.A.), were maintained under standard cell culture conditions at 37 °C in medium containing 10% (v/v) foetal bovine serum. Cells were grown on polycarbonate membranes in Transwells (6.5 mm or 24 mm diameter; Costar, Cambridge, MA, U.S.A.), and experiments were performed on 12–14 days (6.5 mm diameter wells) or 20–22 days (24 mm diameter wells) after seeding.

Treatment with oxidative stress

Confluent monolayers were bathed in Dulbecco's PBS containing 1.2 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.6% (v/v) BSA. Oxidative stress was induced by administering (to both apical and basal compartments) a mixture of xanthine oxidase (20 m-units/ml) and xanthine (0.25 mM) (XO + X) with or without genistein (300 μM). Control cell monolayers were incubated in PBS without XO + X or genistein.

Measurement of transepithelial electrical resistance (TER)

TER was measured according to the method of Hidalgo et al. [29] using a Millicell-ERS Electrical Resistance System (Millipore, Bedford, MA, U.S.A.). TER was calculated as $\Omega \cdot cm^2$ by multiplying the resistance by the surface area of the monolayer (0.33 cm^2). The resistance of the supporting membrane in Transwells (which is usually around 30 $\Omega \cdot cm^2$) was subtracted from all readings prior to calculations.

Unidirectional flux of mannitol

Cell monolayers in Transwells were incubated under different experimental conditions in the presence of 0.2 μCi/ml of D-[2-^{3}H]mannitol (15 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA, U.S.A.) in the basal well. At different times after oxidant administration, 100 μl each of apical and basal media were withdrawn and radioactivity was counted in a scintillation counter. The flux into the apical well was calculated as the percentage of total isotope administered into the basal well per hour per cm^2 of surface area.

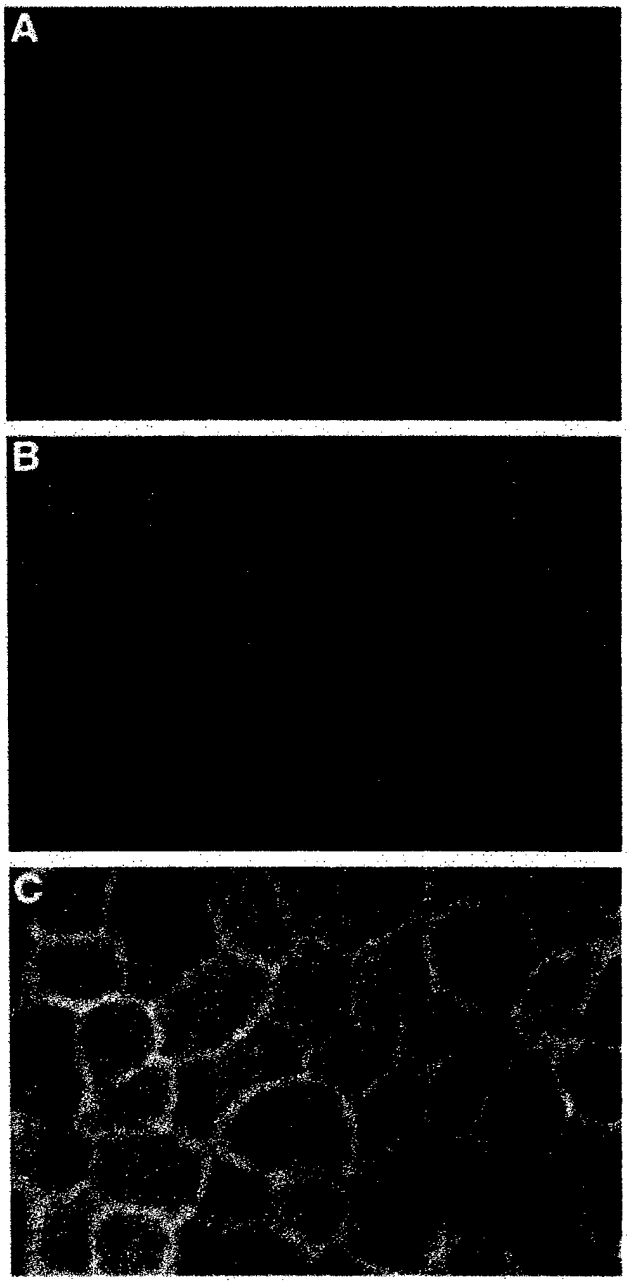

Figure 1 Immunofluorescence localization of XO + X-induced protein tyrosine phosphorylation

Caco-2 cell monolayers incubated with XO + X for 0 min (**A**), 15 min (**B**) or 30 min (**C**) were fixed and stained for phosphotyrosine using FITC-conjugated anti-phosphotyrosine antibody. Immunofluorescence was analysed by confocal laser scanning microscopy.

Immunofluorescence microscopy

Under different experimental conditions, Caco-2 cell monolayers (6.5 mm diameter wells) were washed in PBS and fixed in acetone/methanol (1:1) at 0 °C for 5 min. Cell monolayers were

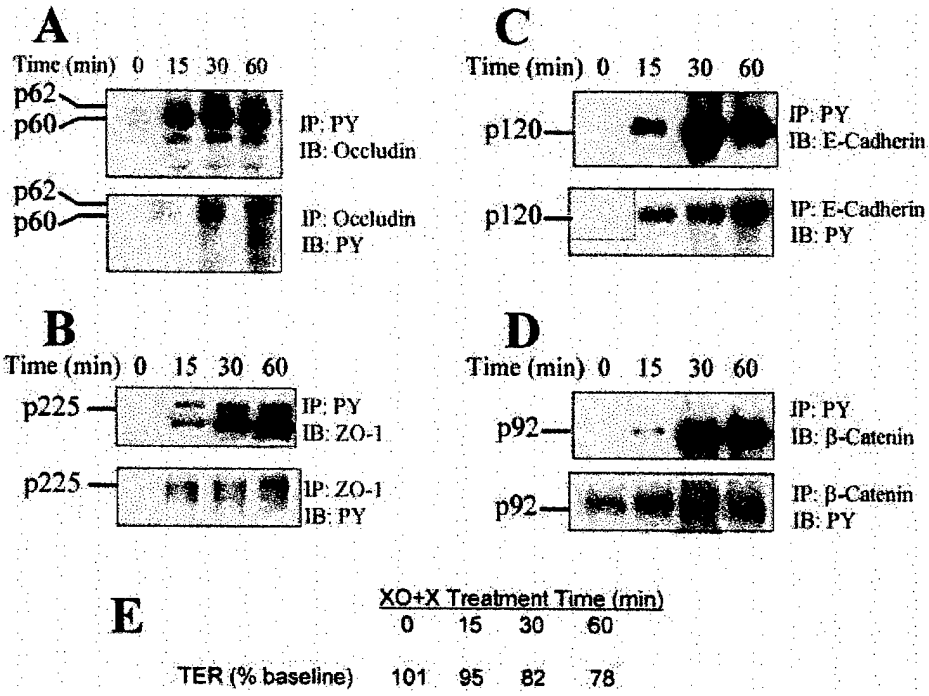

Figure 2 XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin

Caco-2 cell monolayers were treated with XO + X for various times, as indicated. Proteins were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using a biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) and β-catenin (**D**) by immunoblot analysis using rabbit polyclonal anti-occludin and anti-(ZO-1), and mouse monoclonal anti-(E-cadherin) and anti-(β-catenin) antibodies. Alternatively, junctional proteins were immunoprecipitated followed by Western blot analysis for phosphotyrosine. Values in (**E**) represent the mean TER of the cell monolayers that were used. IB, immunoblotting; IP, immunoprecipitation; PY, phosphotyrosine.

blocked in TBST/BSA [20 mM Tris, pH 7.2, 150 mM NaCl and 1% (v/v) BSA] and incubated with primary antibodies for 1 h, followed by incubation for 1 h with secondary antibodies (FITC-conjugated anti-mouse IgG and Cy3-conjugated anti-rabbit IgG antibodies). For staining of phosphotyrosine, cell monolayers were incubated directly with FITC-conjugated anti-phosphotyrosine antibody. Membranes were excised and mounted on a slide. The fluorescence was examined using a confocal laser scanning microscope (Bio-Rad MRC1024, Hercules, CA, U.S.A.), and series of images from 1 μm Y-sections were collected by using comos (confocal microscope operating system). Images were stacked using the software, Confocal Assistant 4.02, and processed by Adobe Photoshop (Adobe Systems Inc., San Jose, CA, U.S.A.). For vertical images, confocal Z-sections were analysed.

Preparation of plasma membrane fractions

Caco-2 cell monolayers (24 mm diameter wells) were washed twice with ice-cold PBS, and once with lysis buffer F (PBS containing 10 mM β-glycerophosphate, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A and 1 mM benzamidine and 1 mM PMSF). Cells were dispersed by homogenization in a Teflon/glass Dounce homogenizer with 50 strokes and lysed by sonication at 4 °C for two strokes (5 s each) with a 30 s interval. The cell lysate was centrifuged first at 3000 *g* for 10 min at 4 °C to sediment the cell debris. The supernatant was centrifuged further at 30000 *g* for 30 min at 4 °C. The pellet was suspended in 500 μl of lysis buffer F. After withdrawal of aliquots (2, 5 and 10 μl for high-density Triton-insoluble, low-density Triton-insoluble and Triton-soluble fractions respectively) for protein assay, the membrane fraction was mixed with an equal volume of 2 × Laemmli's sample buffer and heated at 100 °C for 5 min.

Preparation of cytoskeletal fractions

Caco-2 cell monolayers (24 mm diameter wells) were washed twice with ice-cold PBS, and incubated for 5 min with lysis buffer CS (Tris buffer containing 1.0% Triton X-100, 2 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml bestatin, 10 μg/ml pepstatin-A, 1 mM vanadate and 1 mM PMSF). Extracts were centrifuged first at 15600 *g* for 4 min at 4 °C to sediment the high-density actin cytoskeleton. The supernatant was further centrifuged at 100000 *g* for 4 h at 4 °C to sediment low-density membrane cytoskeleton [30]. The pellet was suspended in 200 μl of lysis buffer CS. After withdrawal of aliquots for protein assay, cytoskeletal fractions were mixed with an equal volume of 2 × Laemmli's sample buffer and heated at 100 °C for 5 min.

Immunoprecipitation

After XO + X treatment, Caco-2 cell monolayers (24 mm diameter wells) were washed with ice-cold 20 mM Tris (pH 7.4) and the proteins were extracted in lysis buffer N (20 mM Tris, pH 7.4, containing 150 mM NaCl, 0.5% Nonidet P40 and proteinase inhibitors as described above for lysis buffer F) at 4 °C for 30 min. Each cell monolayer was extracted in 0.75 ml of lysis buffer N, and extracts from two monolayers were pooled for each value for each experimental condition. Extracts were centrifuged at 3000 *g* for 10 min at 4 °C, and the supernatant,

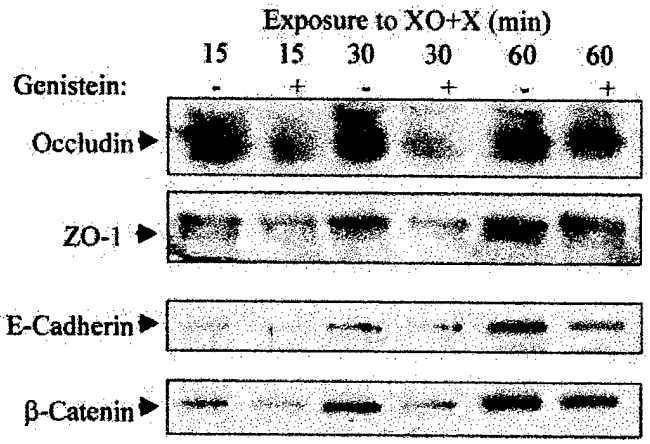

Figure 3 Effect of genistein on XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin

Caco-2 cell monolayers were treated with XO + X with or without genistein for various times. Proteins were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin, ZO-1, E-cadherin and β-catenin by immunoblot analysis using rabbit polyclonal anti-occludin and anti-(ZO-1), and mouse monoclonal anti-(E-cadherin) and anti-(β-catenin) antibodies.

containing 1.0–1.5 mg protein/ml, was incubated with 2 μg of anti-occludin, anti-(ZO-1), anti-(E-cadherin) or anti-(β-catenin) antibodies at 4 °C for 15 h. Immune complexes were isolated by precipitation using protein-A Sepharose or protein-G Sepharose (for 1 h at 4 °C). Washed beads were suspended in 20 μl of Laemmli's sample buffer and heated at 100 °C for 5 min. Extracts were immunoblotted for occludin, claudin-1, ZO-1, E-cadherin or β-catenin, as described below, using specific primary and HRP-conjugated secondary antibodies.

For tyrosine-phosphorylation studies, proteins from whole cell or cytoskeletal fractions were extracted in lysis buffer D (0.3% SDS in 10 mM Tris buffer, pH 7.4, containing 1 mM vanadate and 0.33 mM PMSF) under denaturing conditions (heating at 100 °C for 5 min). Phosphotyrosine was immunoprecipitated as described above using biotin-conjugated anti-phosphotyrosine antibody. Immune complexes were isolated by precipitation using streptavidin-agarose. Immunoprecipitates were immunoblotted for occludin, ZO-1, E-cadherin and β-catenin using antibodies, as described above. Alternatively, junctional proteins were immunoprecipitated using specific antibodies, followed by Western blot analysis for phosphotyrosine.

Immunoblot analysis

Proteins were separated by SDS/PAGE (7.5% gels) and transferred to nitrocellulose PVDF membranes. Membranes were blotted for occludin, ZO-1, E-cadherin and β-catenin using anti-occludin, anti-(ZO-1), anti-(E-cadherin) and anti-(β-catenin) antibodies in combination with HRP-conjugated anti-mouse IgG or HRP-conjugated anti-rabbit IgG antibodies. The blot was developed using the enhanced chemiluminescence method (ECL®, Amersham, Arlington Heights, IL, U.S.A.).

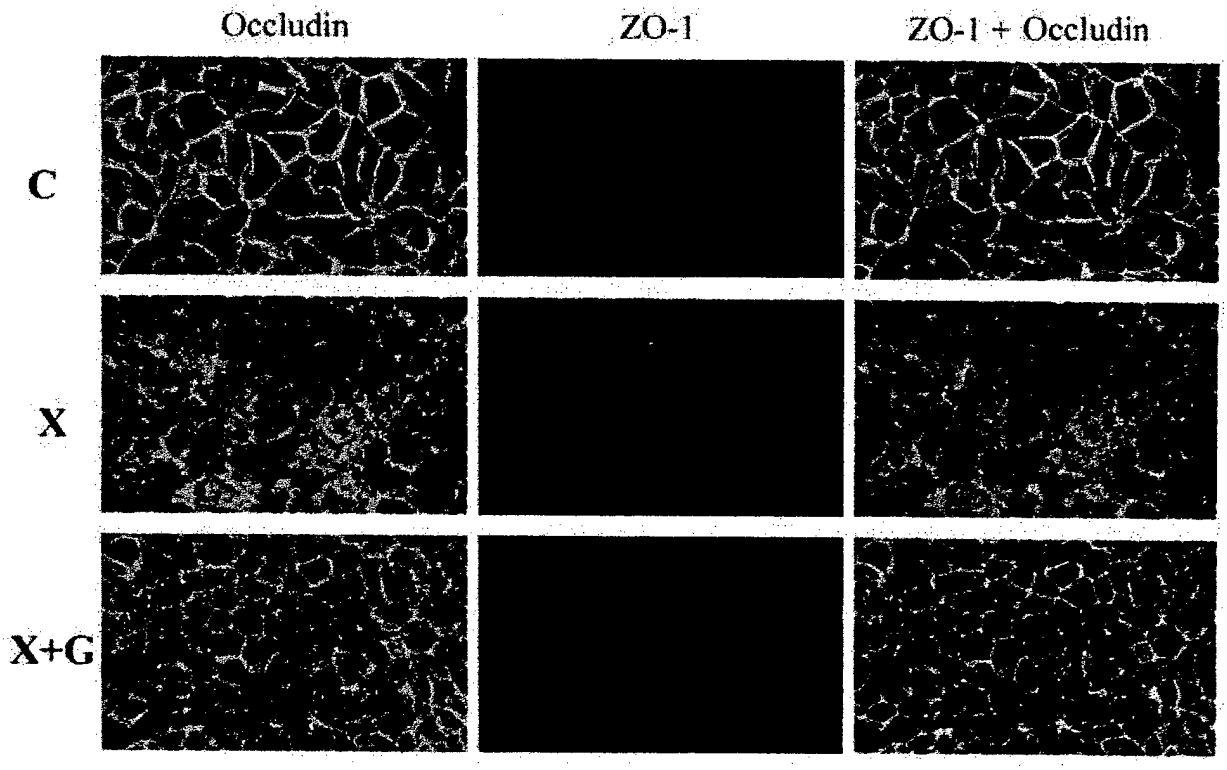

Figure 4 Effect of XO + X on immunofluorescence localization of occludin and ZO-1: Y-sections

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (XG) genistein. Following incubation, cell monolayers were fixed and double-labelled for occludin (green) and ZO-1 (red) by immunofluorescence staining, as described in the Experimental section, using mouse monoclonal anti-occludin and rabbit polyclonal anti-(ZO-1) antibodies.

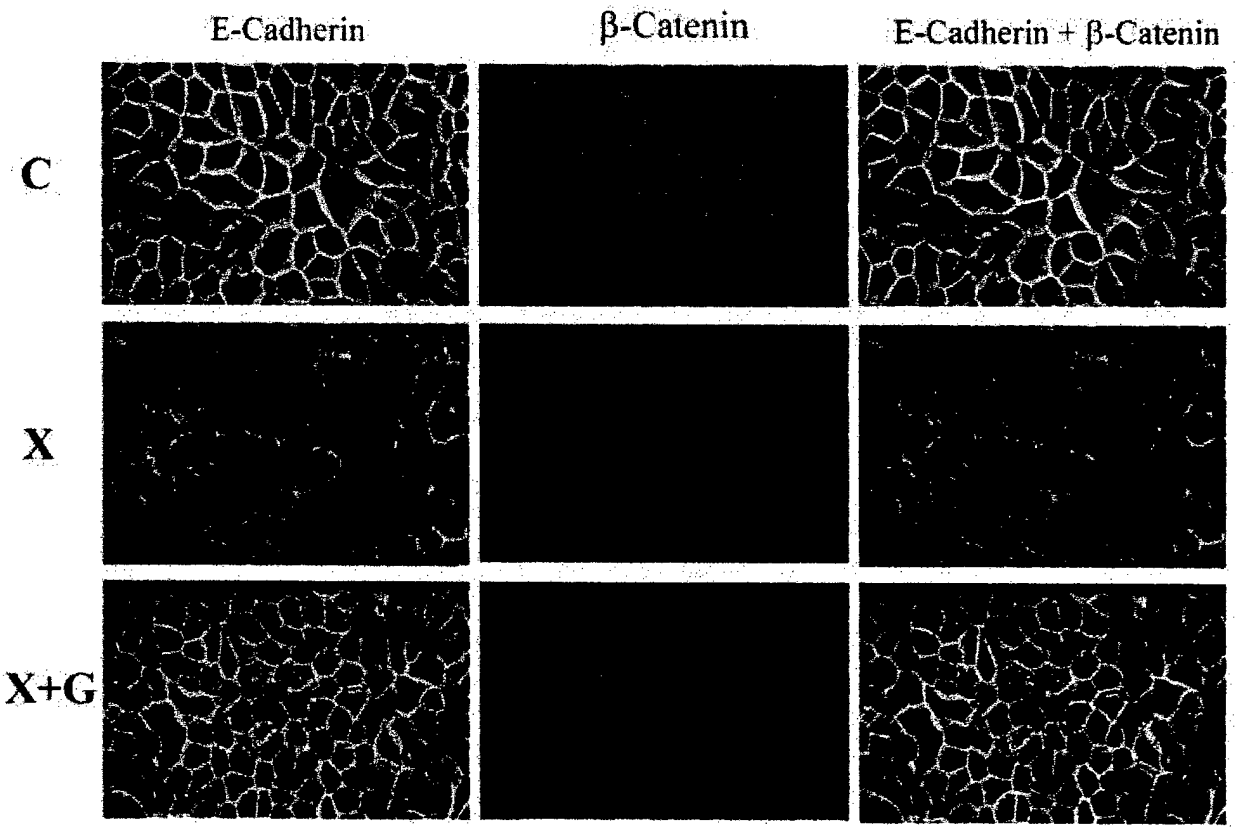

Figure 5 Effect of XO + X on immunofluorescence localization of E-cadherin and β-catenin: Y-sections

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (X + G) genistein. Following incubation, cell monolayers were fixed and double-labelled for E-cadherin (green) and β-catenin (red) by immunofluorescence staining, as described in the Experimental section, using mouse monoclonal anti-(E-cadherin) and rabbit polyclonal anti-(β-catenin) antibodies.

Statistics

Comparison between two groups was performed using Student's *t* test for grouped data in Table 1. The significance in all tests was derived at the 95% or greater confidence level.

RESULTS

Oxidative stress induces tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin

Administration of XO + X into the buffer that incubated the Caco-2 cell monolayer resulted in a time-dependent decrease in TER and increase in mannitol permeability (Table 1). These changes in TER and mannitol permeability were prevented by co-administration of genistein, a tyrosine kinase inhibitor. Immunofluorescence staining of phosphotyrosine and confocal microscopy showed no detectable level of phosphotyrosine in the control cell monolayer (Figure 1), but there was a time-dependent increase in the fluorescence in XO + X-treated cell monolayers. The phosphotyrosine fluorescence was predominantly localized at the intercellular junctions. Immunoprecipitation of phosphotyrosine from denatured extracts of cell monolayers, followed by immunoblot analysis for specific junctional proteins, did not detect any tyrosine-phosphorylated occludin, ZO-1, E-cadherin or β-catenin in control cell monolayers (Figure 2). XO + X treatment resulted in a time-dependent increase in the levels of tyrosine-phosphorylated occludin, ZO-1, E-cadherin and β-catenin. Tyrosine phosphorylation of these proteins was detected as early as 15 min after XO + X administration and peak phosphorylation was achieved by 30 min. The presence of genistein, a tyrosine kinase inhibitor, reduced the XO + X-induced tyrosine phosphorylation of ZO-1, occludin, E-cadherin and β-catenin (Figure 3).

Redistribution of occludin, ZO-1, E-cadherin and β-catenin by oxidative stress by a tyrosine-kinase-dependent mechanism

Immunofluorescence staining and confocal microscopy showed a co-localization of occludin and ZO-1 at the intercellular junctions of control cell monolayers (Figure 4). In XO + X-treated cell monolayers, fluorescence for occludin and ZO-1 was low at the intercellular junctions, while it was increased in the intracellular compartment. There was a partial disruption of the co-localization of occludin and ZO-1. The XO + X-induced redistribution of occludin and ZO-1 was decreased, or absent, in cell monolayers treated with XO + X in the presence of genistein. The fluorescence for E-cadherin and β-catenin was also co-localized at the intercellular junctions of control cell monolayers (Figure 5). In XO + X-treated cell monolayers, the fluorescence for E-cadherin and β-catenin at the intercellular junctions was less than that in control cell monolayers, however E-cadherin and β-catenin appear to be co-localized. Localization of E-cadherin and β-catenin at the intercellular junction in cell

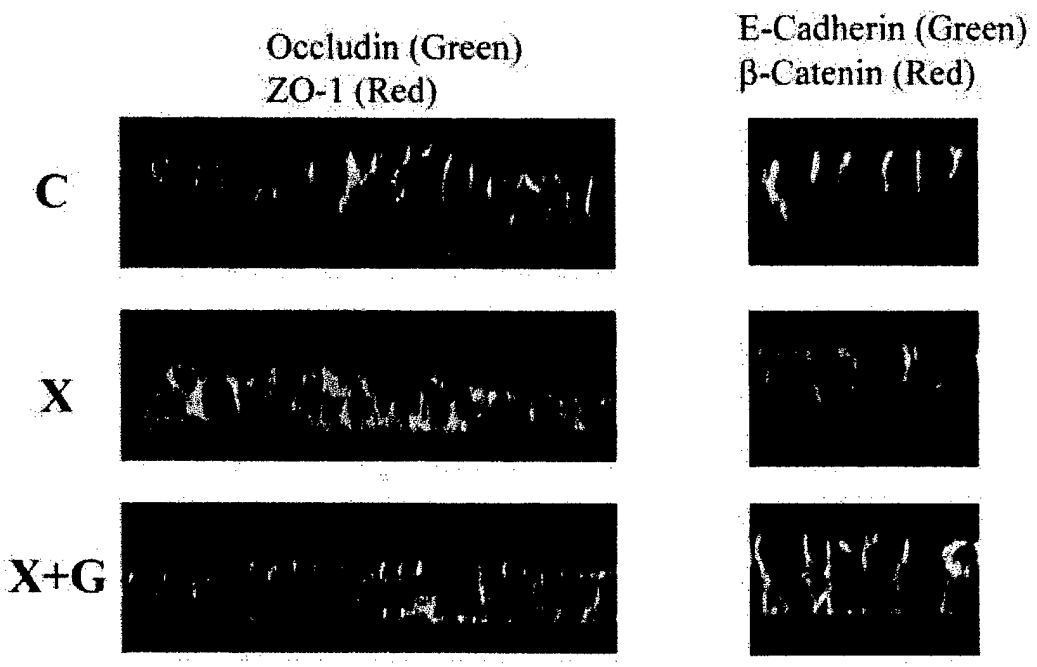

Figure 6 Effect of XO + X on immunofluorescence localization of occludin–ZO-1 and E-cadherin–β-catenin complexes: Z-sections

Caco-2 cell monolayers were incubated for 3 h with PBS alone (C) or PBS containing XO + X without (X) or with (X + G) genistein. Following incubation, cell monolayers were fixed and double-labelled for occludin (green) and ZO-1 (red) or for E-cadherin (green) and β-catenin (red) by immunofluorescence staining as described in the Experimental section. Fluorescence in Z-sections was examined by laser scanning confocal microscopy.

monolayers treated with XO + X and genistein was similar to that in control cell monolayers.

The Z-sections of fluorescence also showed a co-localization of ZO-1 and occludin at the apical end of intercellular junctions (Figure 6). The distribution of ZO-1 and occludin at the apical end of the junctions appeared to be disrupted and redistributed to the lateral and basal parts of the junctions in XO + X-treated cell monolayers. Similarly, the fluorescence for E-cadherin and β-catenin was also localized at the intercellular junctions, with a greater distribution at the apical half of cell junctions (Figure 6). The distribution of E-cadherin and β-catenin at the junction was disrupted in XO + X-treated cell monolayer; however, these two proteins continued to be co-localized. The junctional distribution in cell monolayers co-treated with XO + X and genistein was similar to that in control cell monolayers, except for a partial redistribution of occludin that remained unaffected by genistein.

Oxidative stress disrupts the occludin–ZO-1 complex, but not the E-cadherin–β-catenin complex

To determine the effect of oxidative stress on binding of ZO-1 to occludin and β-catenin to E-cadherin, we analysed co-immunoprecipitation of these protein complexes. Immunoprecipitation of occludin from native extracts of control cell monolayers co-precipitated ZO-1 (Figure 7A). There was a decreased level of ZO-1 detected in anti-occludin immunoprecipitates prepared from XO + X-treated cell monolayers. However, ZO-1 precipitation with anti-occludin immunoprecipitates prepared from cell monolayers that were treated with XO + X and genistein was similar to that in control cell monolayers. Similarly, occludin was detected in immunoprecipitates of ZO-1 in control cell monolayers (Figure 7B). XO + X treatment decreased co-precipitation of occludin with ZO-1, which was prevented by genistein. Immunoprecipitation of E-cadherin co-precipitated β-catenin (Figure 7C), and immunoprecipitation of β-catenin co-precipitated E-cadherin (Figure 7D). However, co-precipitation of E-cadherin and β-catenin was no different in cell monolayers treated with XO + X in the presence or absence of genistein.

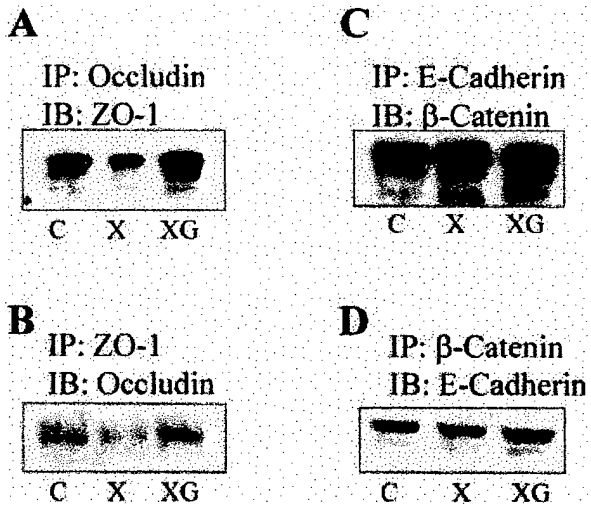

Figure 7 Co-immunoprecipitation of occludin–ZO-1 and E-cadherin–β-catenin complexes

Caco-2 cell monolayers were incubated without (C) or with XO + X in the absence (X) or presence (X + G) of genistein for 3 h. Proteins were extracted under native conditions and immunoprecipitated for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) or β-catenin (**D**) using specific antibodies and immunoblotted for ZO-1, occludin, β-catenin or E-cadherin respectively. IB, immunoblotting; IP, immunoprecipitation.

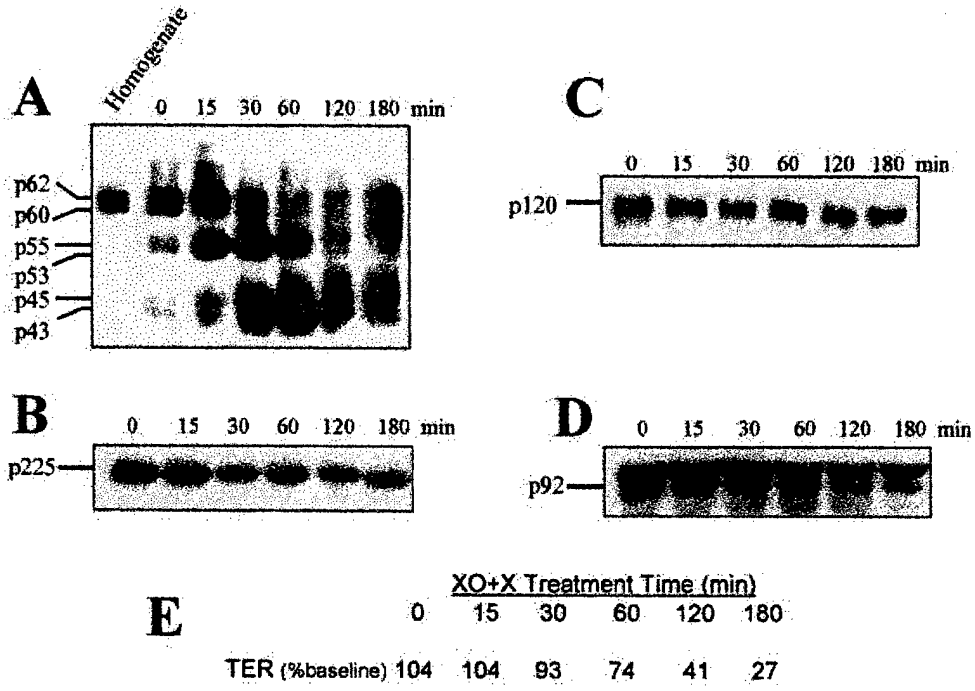

Figure 8 Immunoblot analysis of occludin, ZO-1, E-cadherin and β-catenin in plasma membranes

Caco-2 cell monolayers were treated with XO+X for various times, as indicated. Following treatment, plasma membranes were isolated and analysed by immunoblotting for occludin (**A**), ZO-1 (**B**), E-cadherin (**C**) and β-catenin (**D**), as described in the Experimental section, using mouse monoclonal anti-occludin, rabbit polyclonal anti-(ZO-1), mouse monoclonal anti-(E-cadherin) and rabbit polyclonal anti-(β-catenin) antibodies. The left-hand lane in (**A**) represents the occludin bands in the whole homogenate of untreated cell monolayers. Values in (**E**) represent the mean TER of cell monolayers used in this study.

Oxidative stress induces proteolytic degradation of occludin and a partial reduction of E-cadherin and β-catenin in the plasma membrane fraction

Occludin and E-cadherin are transmembrane proteins, and ZO-1 and β-catenin bind to intracellular domains of occludin and E-cadherin respectively. The localization of occludin, ZO-1, E-cadherin and β-catenin in plasma membranes isolated from the control cell monolayers and the cell monolayers treated with XO+X was determined by immunoblot analysis. Occludin bands with molecular masses of 60 and 62 kDa were detected in plasma membrane fractions of control cell monolayer (Figure 8A). The intensity of this occludin band was reduced by XO+X treatment in a time-dependent manner. The XO+X-mediated decrease of the occludin band was associated with a corresponding appearance of new bands of occludin with molecular masses of 53 and 55 kDa, and 43 and 45 kDa. The intensity of low-molecular mass occludin bands appeared to be much greater than the high-molecular-mass occludin bands. This increased intensity of occludin bands may have been caused by a greater sensitivity of the antibody for binding to the dephosphorylated form of occludin (http://www.zymed.com; Zymed Laboratories, South San Francisco, CA, U.S.A.). A previous study has shown that occludin is highly phosphorylated at serine/threonine residues under basal conditions, and undergoes extensive dephosphorylation during the disruption of TJs [31]. ZO-1 was present in the plasma membrane fractions, and the intensity of this band was unaffected by XO+X treatment (Figure 8B). E-Cadherin (Figure 8C) and β-catenin (Figure 8D) were also present in the plasma membrane fractions of control cell monolayers. XO+X treatment induced only a slight decline in the plasma membrane-associated E-cadherin and β-catenin at 2 h or 3 h treatment.

Although genistein prevented XO+X-induced decrease in TER, it failed to prevent the XO+X-induced decrease in the molecular mass of occludin (Figure 9A). A slight decrease in plasma-membrane-associated E-cadherin and β-catenin by XO+X was also unaffected by genistein (Figure 9A). In the soluble fraction, the amount of ZO-1 was found to be greater in XO+X-treated cell monolayers when compared with that in control cell monolayers. Genistein prevented this increase in soluble ZO-1 by XO+X (Figure 9B). The amounts of E-cadherin and β-catenin in the soluble fraction of cells were not altered by XO+X or genistein. Similar to plasma membrane-associated occludin, soluble-fraction-associated occludin was also transformed to low-molecular-mass forms by XO+X, and this effect of XO+X was not prevented by genistein (Figure 9B).

Treatment of cell monolayers with XO+X in the presence of 1 mM 1,10-phenanthroline, a metalloproteinase inhibitor, prevented the XO+X-induced degradation of occludin in both plasma membrane and soluble fractions (Figure 9). However, 1,10-phenathroline strongly potentiated the effect of XO+X on TER (Figure 9C). It also decreased the plasma-membrane-associated ZO-1 and occludin, but had no effect on E-cadherin and β-catenin in plasma membrane or soluble fractions.

Oxidative stress induces dissociation of cytoskeleton-bound occludin, ZO-1, E-cadherin and β-catenin by a tyrosine-kinase-dependent mechanism

ZO-1 is suggested to link transmembrane occludin with the actin cytoskeleton by binding to both occludin and F-actin [5]. Similarly, β-catenin links E-cadherin with F-actin [8]. To de-

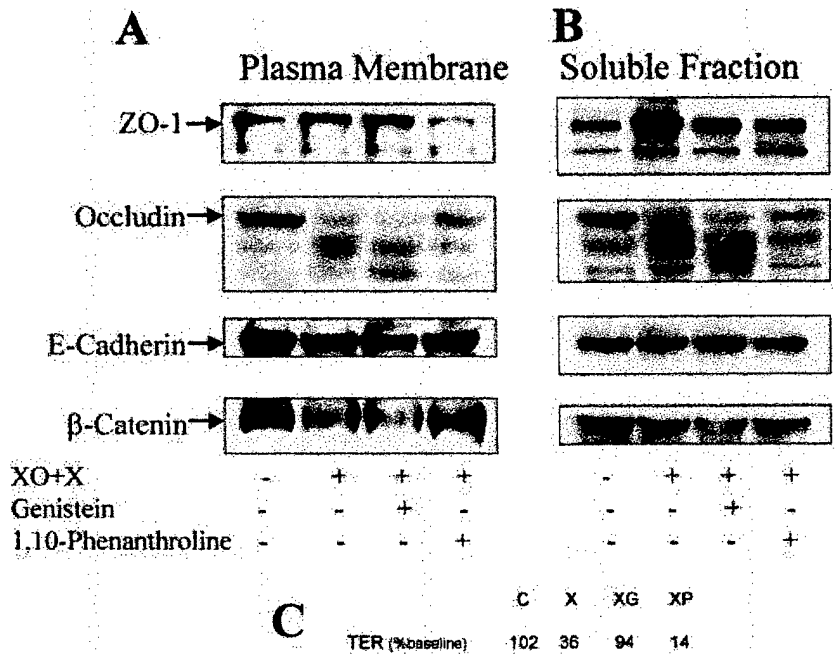

Figure 9 Effect of genistein and 1,10-phenanthroline on XO + X-induced changes in plasma-membrane-associated ZO-1, occludin, E-cadherin and β-catenin

Caco-2 cell monolayers were treated with XO + X in the presence or absence of genistein or 1,10-phenathroline. Control monolayers received no XO + X or genistein. Plasma membranes (**A**) and soluble fractions (**B**) were isolated after 3 h and examined for different proteins by immunoblot analysis. Results in (**C**) show mean TER values.

termine the effect of oxidative stress on cytoskeletal association of TJ and AJ proteins, we analysed the association of different junctional proteins with Triton-insoluble fractions in control and XO + X-treated cell monolayers. High-density Triton-insoluble fractions (cytoplasmic cytoskeleton) were rich in actin, whereas low-density Triton-insoluble fractions (membrane cytoskeleton) were rich in talin, as previously described [30]. High levels of occludin, ZO-1, E-cadherin and β-catenin were detected in the cytoplasmic cytoskeleton of control cell monolayers (Figure 10). XO + X treatment resulted in almost a complete depletion of occludin, and a partial decrease of ZO-1 in the cytoplasmic cytoskeletal fraction. This effect of XO + X was completely prevented by the presence of genistein. XO + X treatment also partially decreased cytoskeleton-bound E-cadherin and β-catenin, which was prevented by the presence of genistein.

Occludin, ZO-1, E-cadherin and β-catenin were also detected in the membrane cytoskeleton fraction (Figure 10). ZO-1, E-cadherin and β-catenin in the membrane cytoskeleton fraction were not affected by XO + X, but were slightly decreased by genistein. Occludin in both the membrane cytoskeleton and Triton-soluble fractions was degraded to low-molecular-mass forms by XO + X treatment (Figure 10). Genistein did not prevent XO + X-induced degradation of occludin in either the membrane cytoskeleton or Triton-soluble fraction. The amount of ZO-1 in the Triton-soluble fraction was greater in XO + X-treated cell monolayers than in control cell monolayers or cell monolayers treated with XO + X in the presence of genistein. E-Cadherin and β-catenin in Triton-soluble fractions were not affected by XO + X or genistein.

Immunoprecipitation of phosphotyrosine in Triton-soluble and insoluble fractions, followed by Western blot analysis for junctional proteins, shows that XO + X treatment induced tyrosine phosphorylation of ZO-1 and β-catenin associated with cytoplasmic actin cytoskeleton (Figure 11). Actin-associated E-cadherin was only slightly phosphorylated, while actin-associated occludin was phosphorylated in the absence of XO + X, which was decreased by treatment with XO + X. This decrease in tyrosine-phosphorylated occludin from the actin cytoskeleton may have been caused by the release of occludin from the cytoskeleton by oxidative stress. XO + X induced a time-dependent increase in tyrosine phosphorylation of occludin associated with membrane cytoskeleton, but only a transient increase in tyrosine phosphorylation of ZO-1, E-cadherin and β-catenin. In the Triton-soluble fraction, XO + X increased tyrosine phosphorylation of all proteins, predominantly that of E-cadherin.

DISCUSSION

A significant body of evidence suggests that intracellular signalling pathways have an important role in regulation of the structure and function of TJs and AJs. Our studies [16,17] have demonstrated that oxidative stress increases paracellular permeability of Caco-2 cell monolayer by a mechanism involving protein tyrosine phosphorylation. In the present study, we show that oxidative-stress-induced increase in paracellular permeability is associated with tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin. Moreover, oxidative stress disrupts occludin–ZO-1 and E-cadherin–β-catenin complexes from the intercellular junctions and their association with cytoskeleton by a tyrosine-kinase-dependent mechanism. Dissociation from the cytoskeleton and redistribution of occludin–ZO-1 and E-cadherin–β-catenin complexes indicate that oxidative stress disrupts TJs and AJs of the Caco-2 cell monolayer by protein tyrosine phosphorylation. Tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin suggests that tyrosine phosphorylation of these proteins may have a role in the mechanism of oxidative-stress-induced dissociation of occludin–

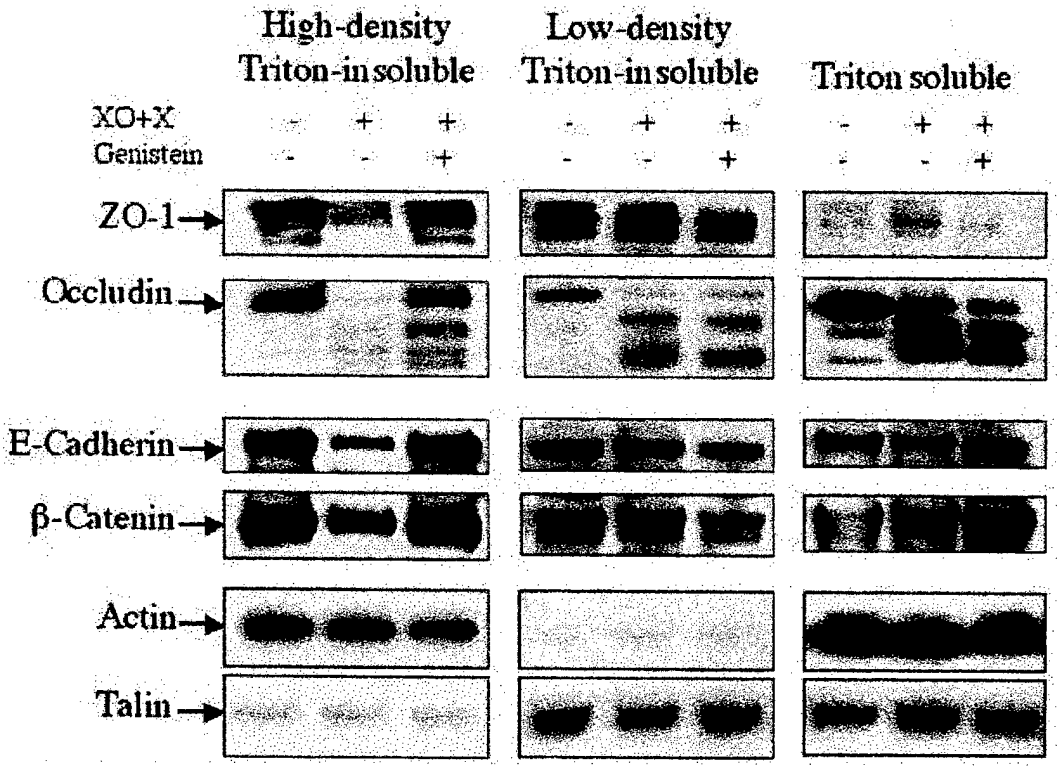

Figure 10 Effect of XO + X and genistein on the association of ZO-1, occludin, E-cadherin and β-catenin in high density Triton-insoluble, low density Triton-insoluble and Triton-soluble fractions

Caco-2 cell monolayers were treated for 3 h with XO + X in the presence or absence of genistein. Control monolayers received no XO + X or genistein. High-density Triton-insoluble (cytoplasmic cytoskeleton), low-density Triton-insoluble (membrane cytoskeleton) and Triton-soluble fractions were isolated and examined for different proteins by immunoblot analysis. Actin and talin were analysed as markers for cytoplasmic cytoskeleton and membrane cytoskeleton fractions respectively.

ZO-1 and E-cadherin–β-catenin complexes from the cytoskeleton and disruption of TJs and AJs.

Our previous study demonstrated that oxidative stress induces a rapid increase in tyrosine phosphorylation of clusters of proteins in Caco-2 cell monolayers [16]. However, the identity of any of these proteins is not known. Immunofluorescence localization of phosphotyrosine in the present study confirms an oxidative-stress-induced increase in protein tyrosine phosphorylation. The location of phosphotyrosine at the intercellular junctions suggested that oxidative stress phosphorylates the proteins associated with TJs and AJs. The present study demonstrates that oxidative stress induces a rapid increase in tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin. Tyrosine phosphorylation of E-cadherin and β-catenin has been previously shown to prevent the binding of β-catenin to E-cadherin and cadherin-based cell–cell adhesion [28,32,33]. Tyrosine phosphorylation of occludin and ZO-1 suggests that tyrosine phosphorylation may mediate disruption and redistribution of the occludin–ZO-1 complex from TJs, leading to an increase in paracellular permeability. Results also show that genistein, a tyrosine kinase inhibitor, reduces the oxidative-stress-induced tyrosine phosphorylation of junctional proteins.

Immunofluorescence localization of occludin and ZO-1 at the intercellular junctions indicates the presence of well defined TJs in Caco-2 cell monolayers, which contribute to a TER of 400–500 $\Omega \cdot cm^2$. Occludin is the first transmembrane protein localized at the TJ [1]. At the intracellular C-terminal domain, occludin binds to ZO-1, ZO-2 and ZO-3, and the binding of ZO-1 to occludin is well characterized as being required for the maintenance of the integrity of TJs [6]. In the present study, we show that oxidative stress causes a reduction in ZO-1 stain (by immunofluorescence) at the intercellular junctions and a redistribution of occludin from the intercellular junctions. This observation indicates that the interaction between ZO-1 and occludin is disrupted by oxidative stress. Disruption of the occludin–ZO-1 complex was further confirmed by a loss of co-immunoprecipitation of ZO-1 and occludin in oxidative-stress-treated cell monolayer. The prevention of oxidative-stress-induced redistribution and the loss of interaction between occludin and ZO-1 by genistein indicates the role of tyrosine kinase activity in disruption of occludin–ZO-1 complex. Immunofluorescence staining also showed a dissociation and redistribution of the AJ-specific proteins, E-cadherin and β-catenin, from the intercellular junctions. Unlike the occludin–ZO-1 complex, the E-cadherin–β-catenin complex was maintained and redistributed without dissociation. This was confirmed by the lack of effect of oxidative stress on co-immunoprecipitation of E-cadherin and β-catenin.

ZO-1 establishes a link between transmembrane occludin and cytoskeleton [5]. Interaction with the cytoskeleton anchors occludin at the TJ, and this interaction is crucial for the maintenance of the structure and function of TJs. Disruption of cytoskeleton by cytochalasin-D disrupts TJs and increases paracellular permeability [34]. In the present study, we show that occludin and ZO-1 are associated with the isolated cytoskeletal fraction. Oxidative stress almost completely releases occludin

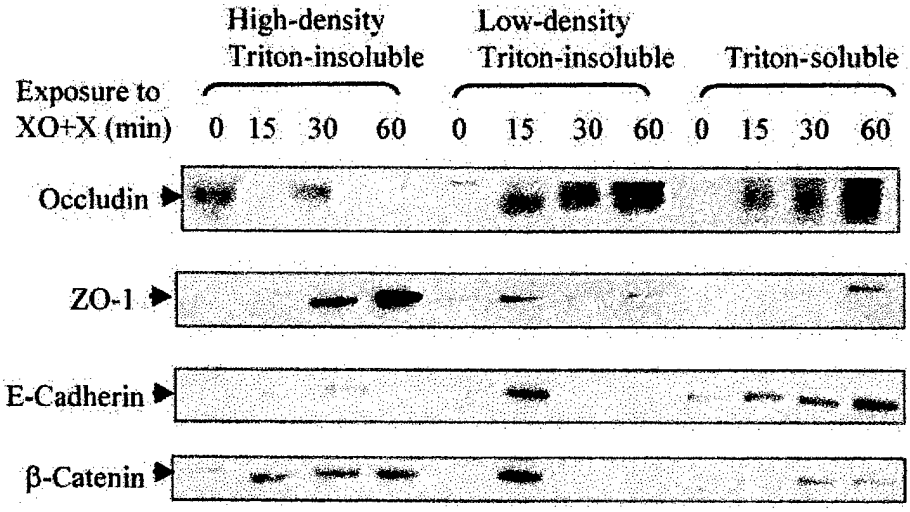

Figure 11 XO + X-induced tyrosine phosphorylation of occludin, ZO-1, E-cadherin and β-catenin in Triton-insoluble and Triton-soluble fractions

Caco-2 cell monolayers were treated with XO + X for various times, as indicated. High-density Triton-insoluble, low-density Triton-insoluble and Triton-soluble fractions were prepared. Proteins in different fractions were extracted under denaturing conditions and immunoprecipitated for phosphotyrosine using a biotin-conjugated anti-phosphotyrosine antibody. Immunoprecipitates were then analysed for occludin, ZO-1, E-cadherin and β-catenin by immunoblot analysis.

from the cytoskeleton and partially dissociates cytoskeletal ZO-1. Similarly, cytoskeleton-associated E-cadherin and β-catenin were partially released by oxidative stress. However, oxidative stress failed to alter plasma-membrane-associated ZO-1, whereas E-cadherin and β-catenin in plasma membrane were only slightly decreased. Although it undergoes proteolytic degradation, the intact occludin and its degradation products continue to be associated with the plasma membrane fraction of oxidative-stress-induced cell monolayers. These observations indicate that oxidative-stress-induced disruption of TJs and AJs is mainly caused by the dissociation of interaction between cytoskeleton and the occludin–ZO-1 and E-cadherin–β-catenin complexes, rather than the release of these proteins from the plasma membrane. A complete prevention of oxidative-stress-induced release of cytoskeleton-associated occludin–ZO-1 and E-cadherin–β-catenin complexes by genistein demonstrates that tyrosine phosphorylation plays a role in disrupting the interaction of occludin–ZO-1 and E-cadherin–β-catenin complexes with the actin cytoskeleton. Oxidative stress induced tyrosine phosphorylation of ZO-1 and β-catenin that are associated with actin cytoskeleton, whereas E-cadherin was only slightly phosphorylated. Occludin in actin cytoskeleton was phosphorylated under basal conditions, whereas its phosphorylation was decreased by oxidative stress. Decrease in tyrosine phosphorylated occludin may have been caused by the release of occludin from the actin cytoskeleton. In the membrane cytoskeleton, oxidative stress induced tyrosine phosphorylation of occludin and transient phosphorylation of ZO-1, E-cadherin and β-catenin. These results suggest that tyrosine phosphorylation of ZO-1 and β-catenin may play a crucial role in the oxidative-stress-induced disruption of occludin–ZO-1 and E-cadherin–β-catenin complexes.

On the other hand, genistein failed to prevent oxidative-stress-induced degradation of occludin or the minor decrease in E-cadherin and β-catenin in the plasma membrane fraction. Interestingly, the decrease in occludin in the plasma membrane fraction was accompanied by a time-dependent appearance of low-molecular-mass occludin bands (43 and 45 kDa, and 53 and 55 kDa). Phenylarsine oxide, an inhibitor of protein tyrosine phosphatases, disrupted TJs of endothelial cell monolayers by proteolytic degradation and redistribution of occludin, but not ZO-1, E-cadherin or β-catenin. Occludin redistribution and TJ permeability by phenylarsine oxide was partially prevented by a metalloproteinase inhibitor [35]. However, in the present study we show that oxidative-stress-induced occludin degradation plays no role in disruption of TJs. The metalloproteinase inhibitor 1,10-phenanthroline prevented the occludin degradation by oxidative stress, but it potentiated the oxidative-stress-induced decrease in TER.

In summary, the present study shows that oxidative stress induces tyrosine phosphorylation of occludin–ZO-1 and E-cadherin–β-catenin complexes, and results in redistribution of these protein complexes in Caco-2 cell monolayers. Tyrosine-kinase-dependent dissociation of occludin–ZO-1 and E-cadherin–β-catenin complexes from the cytoskeleton appears to be the main mechanism involved in oxidative-stress-induced disruption of TJs and AJs, and increase in paracellular permeability.

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Efflux of Dietary Flavonoid Quercetin 4′-β-Glucoside across Human Intestinal Caco-2 Cell Monolayers by Apical Multidrug Resistance-Associated Protein-2[1]

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ABSTRACT

Although there is strong evidence to suggest that flavonoid consumption is beneficial to human health, the extent to which flavonoids are absorbed and the mechanisms involved are controversial. Contrary to common dogma, we previously demonstrated that quercetin 4′-β-glucoside, the predominant form of the most abundant dietary flavonoid, quercetin, was not absorbed across Caco-2 cell monolayers. The aim of this study was to test the hypothesis that a specific efflux transporter is responsible for this lack of absorption. Transport of quercetin 4′-β-glucoside, alone or with inhibitors, was examined with Caco-2 cell monolayers. In addition, subcellular localization of the multidrug resistance-associated proteins MRP1 and MRP2 was examined by immunofluorescent confocal microscopy. Efflux of quercetin 4′-β-glucoside, a saturable process, was not altered by verapamil, a P-glycoprotein inhibitor, but was competitively inhibited by MK-571, an MRP inhibitor. These data in combination with immunofluorescent localization of MRP2 to the apical membrane support a role for MRP2 in the intestinal transcellular efflux of quercetin 4′-β-glucoside. These results suggest a role for MRP2 in the transport of a new class of agents, dietary glucosides.

Flavonoids are highly diverse, low molecular weight polyphenolic compounds. Due to their ubiquitous distribution in plants, humans are constantly exposed to a large variety of flavonoids, but precise exposure levels are not yet known. Although early estimates reported the average intake of all flavonoids to be as high as 1 g/day (Pierpoint, 1986), newer data suggest that the daily intake may be lower. Based on data from The Netherlands, a Western diet contains approximately 16 mg/day of the most commonly consumed flavonoid, quercetin (Hertog et al., 1993). However, very large interindividual variability can be expected based on dietary preferences. Significant sources of quercetin include onions, apples, tea, and red wines.

Epidemiological data have demonstrated an association between a diet that is rich in quercetin and a significant reduction in the risk of mortality from coronary heart disease (Hertog et al., 1993; Knekt et al., 1996) and a reduced risk of stroke (Keli et al., 1996). Quercetin is a potent antioxidant, which chelates metal ions to prevent the Fenton reaction and is capable of scavenging hydroxyl and peroxy radicals (Manach et al., 1996). In addition, quercetin and quercetin monoglucosides have been shown to inhibit 15-lipoxygenase, an enzyme thought to play a role in the oxidative modification of low density lipoprotein, leading to foam cell formation in the early development of atherosclerosis (da Silva et al., 1998).

Epidemiological studies also support an association between dietary flavonoids and a reduced risk of certain cancers, including stomach carcinoma and lung cancer (Dorant et al., 1996; Knekt et al., 1997). Animal studies (Deschner et al., 1993) as well as in vitro studies suggest that the flavonoids exert preventive effects in cancer. Proposed mechanisms for anticancer benefits include numerous effects on signal transduction pathways involved in cell proliferation (Weber et al., 1996; Lepley and Pelling, 1997) and angiogenesis (Fotsis et al., 1997), as well as inhibition of enzymes involved with procarcinogen bioactivation such as cytochrome P450 (Tsyrlov et al., 1994) and sulfotransferase enzymes (Walle et al., 1995).

Although there is strong evidence to suggest beneficial effects of flavonoids in human health, the extent and mechanism by which flavonoids reach the systemic circulation from dietary sources are controversial. Plant flavonoids are predominantly found as β-glycosides with flavonols (including quercetin) existing as 3, 7, and 4′ *O*-glycosides, whereas other flavonoids, such as flavones, flavonones, and isoflavones, are mainly glycosylated at position 7 (Price et al.,

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ABBREVIATIONS: MRP, multidrug resistance-associated protein; MEM, minimum essential medium; TEER, transepithelial electrical resistance.

1997; Fossen et al., 1998). With the notable exception of fermentation and autolysis, which release the aglycone, flavonoid glycosides are relatively resistant to most food preparation methods (Coward et al., 1993). Thus, when consumed, flavonoids are present primarily as glycosides.

An original model of flavonoid bioavailability assumed that flavonoid glycosides were too polar to be absorbed from the small intestine and that absorption was dependent on the cleavage of the β-glycoside linkage by the colonic microflora (Griffiths and Barrow, 1972). Hollman et al. (1995) indirectly calculated the absorption of quercetin aglycone as well as quercetin glucosides from an onion meal in ileostomy patients. Based on the results, they proposed that quercetin glucosides were actively absorbed via the intestinal glucose transporter. In a direct examination of the transcellular absorption of quercetin glucosides with the Caco-2 cell model of human intestinal absorption, in which glucose is very rapidly absorbed, we found that quercetin 4′-β-glucoside was not absorbed, whereas quercetin 3,4′-β-diglucoside demonstrated minimal absorption (Walgren et al., 1998). In fact, contrary to all hypotheses, both of these glucosides demonstrated significant efflux, suggesting that a drug efflux pump may be involved in the basolateral-to-apical transport of these dietary components.

In this study, we have examined the role of known intestinal efflux pumps in the transcellular efflux of quercetin 4′-β-glucoside in human intestinal Caco-2 cell monolayers. Our observations indicate that the multidrug resistance-associated protein MRP2 plays a major role in this efflux. MRP2 identification and subcellular localization to the apical membrane in the Caco-2 cells were established by immunofluorescent confocal microscopy.

Materials and Methods

MK-571 was a generous gift from A. W. Ford-Hutchinson, Merck-Frosst Center for Therapeutic Research, Pointe Claire-Dorval, Quebec, Canada. Dulbecco's PBS with 0.1 g l^{-1} calcium chloride was purchased from Life Technologies (Grand Island, NY). Quercetin 4′-β-glucoside was isolated from the red onion as described previously (Walgren et al., 1998). Verapamil HCl was obtained from Knoll Pharmaceutical (Whippany, NJ). Anti-human MRP1 and MRP2 antibodies were purchased from Kamiya Biomedical (Seattle, WA). Except where noted, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Caco-2 cells obtained from American Type Culture Collection (Rockville, MD) were cultured in Eagle's minimum essential medium (MEM) (Cellgro; Mediatech, Herndon, VA) supplemented with 1% MEM nonessential amino acids (Mediatech), 10% fetal bovine serum (Summit Biotechnology, Fort Collins, CO), 100 U ml^{-1} penicillin, and 0.1 mg ml^{-1} streptomycin and were grown in a humidified atmosphere of 5% CO_2 at 37°C. Cells were subcultured at 80% confluence.

Transport Studies. For all transcellular transport studies, Caco-2 cells were seeded in 12-mm i.d. Transwell inserts (polycarbonate membrane, 0.4-μm pore size; Corning Costar Corp., Cambridge, MA) in 12-well plates at a density of 1.0×10^5 cells cm^{-2}. The basolateral (serosal) and apical (mucosal) compartments contained 1.5 and 0.5 ml of culture medium, respectively. Culture medium was replaced three times a week for 14 days and daily thereafter. Caco-2 cells in Transwells at passage 48 to 93 were used for transport experiments 18 to 25 days post seeding. Inserts with transepithelial electrical resistance (TEER) values >350 Ω cm^2 in culture medium were washed twice for 30 min with warm PBS.

Stock solutions of quercetin 4′-β-glucoside in ethanol were diluted with PBS before transport experiments. The resulting maximum final concentration of ethanol, 0.5%, did not affect TEER values or the transport of mannitol, a marker of paracellular transport. All other compounds were dissolved in transport medium. Transport medium containing substrate was added to either the apical (0.5 ml) or basolateral (1.5 ml) side of the inserts, whereas the receiving chamber contained the corresponding volume of PBS. Where applicable, inhibitors (MK-571, verapamil) were added to both chambers. Upon termination of the 1-h incubation at 37°C, samples were collected for immediate analysis.

Samples containing quercetin 4′-β-glucoside were quantified by reversed phase HPLC on a Millennium HPLC system (Waters Corp., Milford, MA) with a Symmetry C18 column, 3.9 × 150 mm, and a model 996 photodiode array detector. The mobile phase consisted of 35% methanol in 5% acetic acid with a flow rate of 0.9 ml min^{-1}. Quercetin 4′-β-glucoside peak areas were measured at 370 nm.

Calculations and Statistics. Apparent permeability coefficients (P_{app}) were calculated with the following equation:

$$P_{app} = \frac{V}{AC_0}\frac{dC}{dt} = \text{cm s}^{-1}$$

where V = the volume of the solution in the receiving compartment, A = the membrane surface area, C_0 = the initial concentration in the donor compartment, and dC/dt = the change in drug concentration in the receiver solution over time (Artursson and Karlsson, 1991).

Transport data are expressed as a mean flux of five or more determinations ± S.E. ANOVA was used to evaluate differences in flux. A P value <.05 was considered significant. Apparent kinetic constants were obtained by fitting data to models of competitive, noncompetitive, and uncompetitive inhibition (Segal, 1975) with the Solver function in Microsoft Excel 97 (Microsoft, Redmond, WA).

Immunofluorescent Localization of MRP in Caco-2 Cells. Washed confluent monolayers of Caco-2 cells grown on Transwells were fixed at room temperature with 3% paraformaldehyde for 15 min to preserve three-dimensional structure. Cells were then permeabilized for 10 min with 0.1% Triton X-100 and blocked for 30 min with 5% normal goat serum. Inserts were incubated for 1 h with antibodies raised against MRP2 (M_2I-4, M_2III-6) or MRP1 (MRPr1, MRPm6). Primary antibody binding was detected with Alexa 488 goat anti-mouse IgG (MRPm6, M_2I-4, M_2III-6) or fluorescein isothiocyanate anti-rat IgG (MRPr1; Molecular Probes, Eugene, OR). Cells were incubated for 3 min with propidium iodide for counterstaining of nucleic acids. Alternately, fixed Caco-2 monolayers on polycarbonate membranes were cut from Transwells, embedded in O.C.T. Compound (Miles, Elkhart, IN) and frozen with liquid nitrogen. Frozen sections (5 μm) were cut perpendicular to the membrane. Sections were then permeabilized and stained as described above. Stained monolayers and sections were examined with a Bio-Rad MRC 1024 laser scanning confocal microscope (Hercules, CA). To control for nonspecific binding, matching inserts were treated similarly but with the omission of the primary antibody.

Results

Transcellular Efflux of Quercetin 4′-β-Glucoside. Efflux of quercetin 4′-β-glucoside was monitored by a molecularly specific HPLC method at 370 nm as depicted in Fig. 1. Formation of quercetin or quercetin glucuronides was not observed. The latter was a distinct possibility based on a previous study in the rat intestine (Crespy et al., 1999). However, formation of the glucuronides was ruled out based on the absence of peaks at the retention times (5.0, 6.0, and 10.3 min) identified for the three glucuronides formed when quercetin was incubated with human liver microsomes or

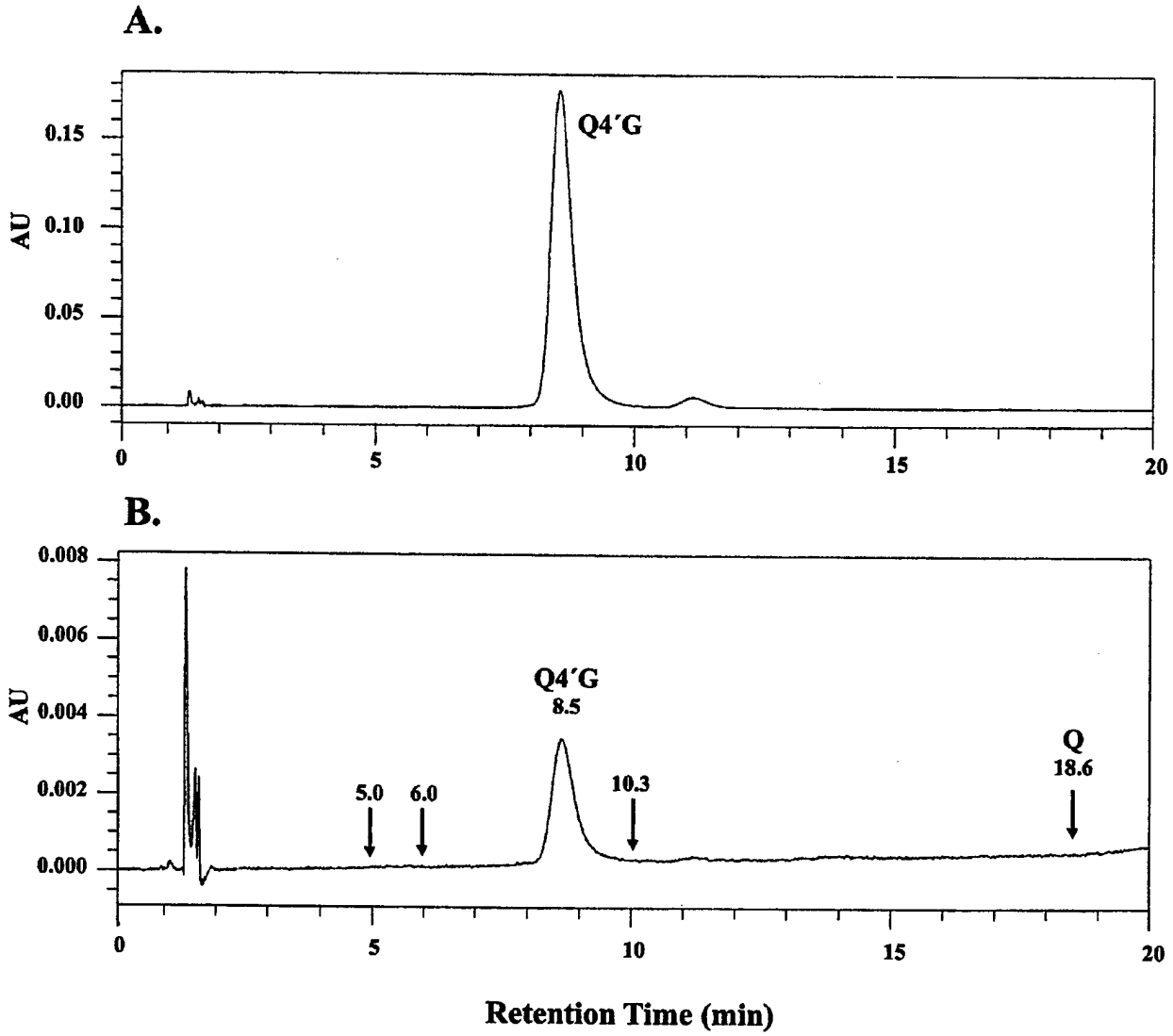

Fig. 1. HPLC of quercetin 4′-β-glucoside in Caco-2 transport experiments. A, loading solution (50 μM). B, apical solution 60 min after loading the basolateral side with quercetin 4′-β-glucoside. The same volume (100 μl) was injected in both tracings. Arrows denote retention times identified for quercetin (Q) and quercetin glucuronides. AU, absorption units; Q4′G, quercetin 4′-β-glucoside.

recombinant human UGT1A9 and the cofactor UDP-glucuronic acid (Y. Otake and T. Walle, unpublished data).

The efflux of quercetin 4′-β-glucoside across Caco-2 cell monolayers was examined for concentration dependence. Transport of quercetin 4′-β-glucoside was determined in the basolateral-to-apical direction over the concentration range of 10 to 300 μM (Fig. 2A). The apparent permeability coefficient decreased significantly with increased concentration over the range examined, from $3.87 \pm 0.40 \times 10^{-6}$ cm s^{-1} at 10 μM to $0.75 \pm 0.06 \times 10^{-6}$ cm s^{-1} at 300 μM, consistent with a saturable secretory mechanism. The solubility of quercetin 4′-β-glucoside was determined to be >300 μM and, thus, did not influence the results shown in Fig. 2A.

To test the hypothesis that a transporter is responsible for the efflux and lack of absorption of quercetin 4′-β-glucoside, we examined the role of P-glycoprotein and the MRPs by measuring efflux in the presence of selective inhibitors (Fig. 2B). Verapamil, an inhibitor of P-glycoprotein, did not significantly alter the flux of quercetin 4′-β-glucoside in the basolateral-to-apical direction, P_{app} of $2.36 \pm 0.09 \times 10^{-6}$ cm s^{-1} in control inserts versus $2.04 \pm 0.23 \times 10^{-6}$ cm s^{-1} with 50 μM verapamil. The presence of 50 μM MK-571, an MRP inhibitor, resulted in a greater than 80% reduction in apparent permeability, $0.40 \pm 0.04 \times 10^{-6}$ cm s^{-1} ($P < .001$). This finding in combination with the previous detection in our laboratory of MRP2 but not MRP1 in Caco-2 cells (Walle et al., 1999b) implicated a role for MRP2 in the transcellular efflux of quercetin 4′-β-glucoside.

Kinetic Properties of Quercetin 4′-β-Glucoside Efflux. To examine the mechanism of inhibition and determine apparent kinetic constants, we examined quercetin 4′-β-glucoside efflux over a range of substrate and MK-571 concentrations, 10 to 300 μM and 0.1 to 50 μM, respectively. Data were acquired in three separate experiments each with triplicate determinations at each substrate and inhibitor concentration. Both transport of quercetin 4′-β-glucoside and inhibition of transport by MK-571 demonstrated concentration dependence (Fig. 3). Data were fitted to one- and two-component models of competitive, noncompetitive, and uncompetitive inhibition with the Solver function in Microsoft Excel 97.

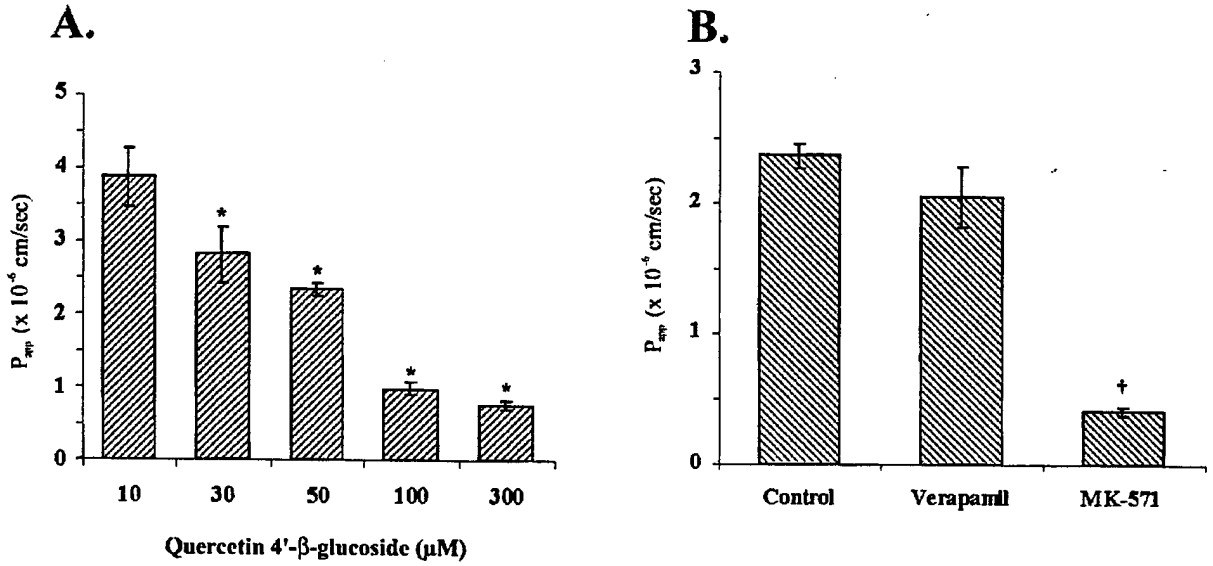

Fig. 2. A, concentration-dependent efflux of quercetin 4′-β-glucoside. Washed Caco-2 cell monolayers were loaded on the basolateral side with quercetin 4′-β-glucoside. Samples were collected for analysis after 1 h. Data represent the mean ± S.E. of 4 to 17 independent observations at each concentration. *$P < .05$, significantly lower than at 10 μM. B, inhibition of quercetin 4′-β-glucoside efflux. Washed Caco-2 cell monolayers were preincubated for 30 min with PBS (control) or with 50 μM verapamil or 50 μM MK-571 in PBS. Buffer ± inhibitor was then replaced and 50 μM quercetin 4′-β-glucoside ± inhibitors was loaded on the basolateral side. Samples were collected for analysis after 1 h. Data represent the mean ± S.E. of five independent observations. †$P < .0001$, significantly less than control experiments.

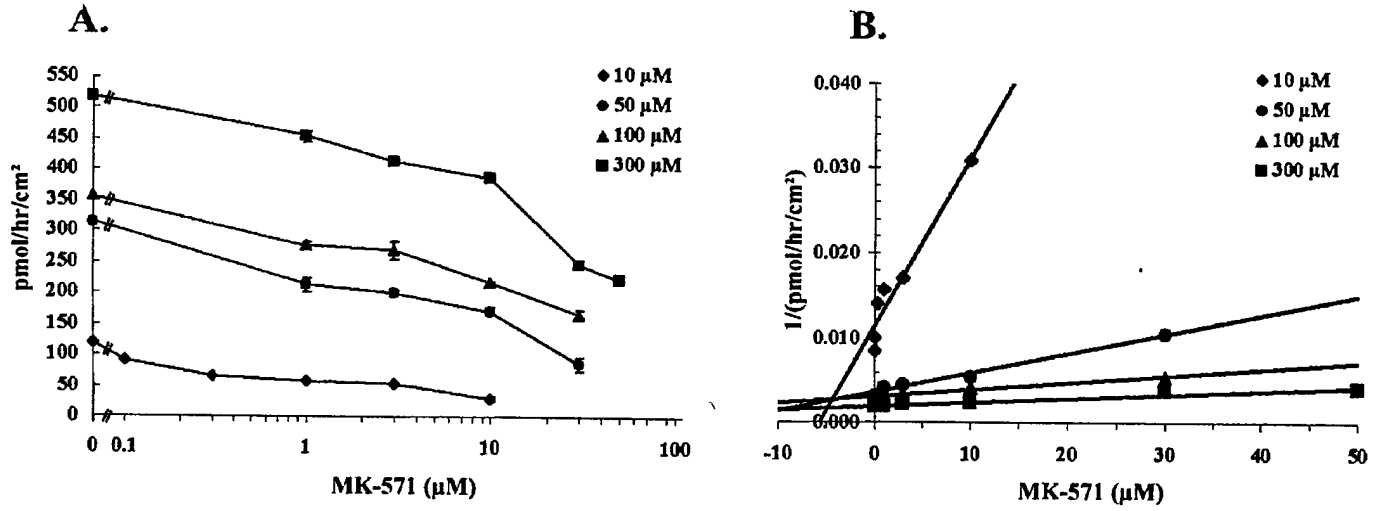

Fig. 3. Concentration-dependent inhibition of quercetin 4′-β-glucoside transport by MK-571. Washed Caco-2 cell monolayers were preincubated for 30 min with MK-571 in PBS. Buffer ± inhibitor was then replaced and quercetin 4′-β-glucoside ± inhibitor was loaded on the basolateral side. Samples were collected for analysis after 1 h. A, data represent the mean flux ± S.E. of triplicates from three independent experiments. B, Dixon plot of data presented in A.

Results obtained with a two-component model were not different from those obtained with a one-component model, and the simpler model was adopted. The best fit was obtained with a one-component model of competitive inhibition. Data are summarized in Table 1.

Transcellular Absorption of Quercetin 4′-β-Glucoside. In our previous studies, we have demonstrated a lack of apical to basolateral absorption of quercetin 4′-β-glucoside across Caco-2 monolayers. Efflux of the monoglucoside across the apical membrane by MRP2 is one potential explanation for this observation. To test this possibility we examined the apical-to-basolateral flux of quercetin 4′-β-glucoside in the presence of 50 μM MK-571. Transport of quercetin 4′-β-glucoside was not observed at substrate concentrations less than 250 μM. A modest but statistically significant ($P < .001$) absorption, $P_{app} = 0.032 \pm 0.008 \times 10^{-6}$ cm s^{-1}, was observed at 250 μM quercetin 4′-β-glucoside in the presence of

TABLE 1

Apparent kinetic constants derived from the concentration dependent inhibition of quercetin 4′-β-glucoside transport by MK-571

The kinetic constants K_m, V_{max}, and K_I were calculated with nonlinear regression analysis using the Solver function in Microsoft Excel 97 (see experiment in Fig. 3). The best fit of the data was obtained with a single component model of competitive inhibition. Data shown represent the mean ± S.E. from three experiments with triplicate determinations.

	K_m	V_{max}	K_I
	μM	pmol h^{-1} cm^{-2}	μM
Quercetin 4′-β-glucoside	43.6 ± 7.4	556 ± 72	
MK-571			5.6 ± 0.9

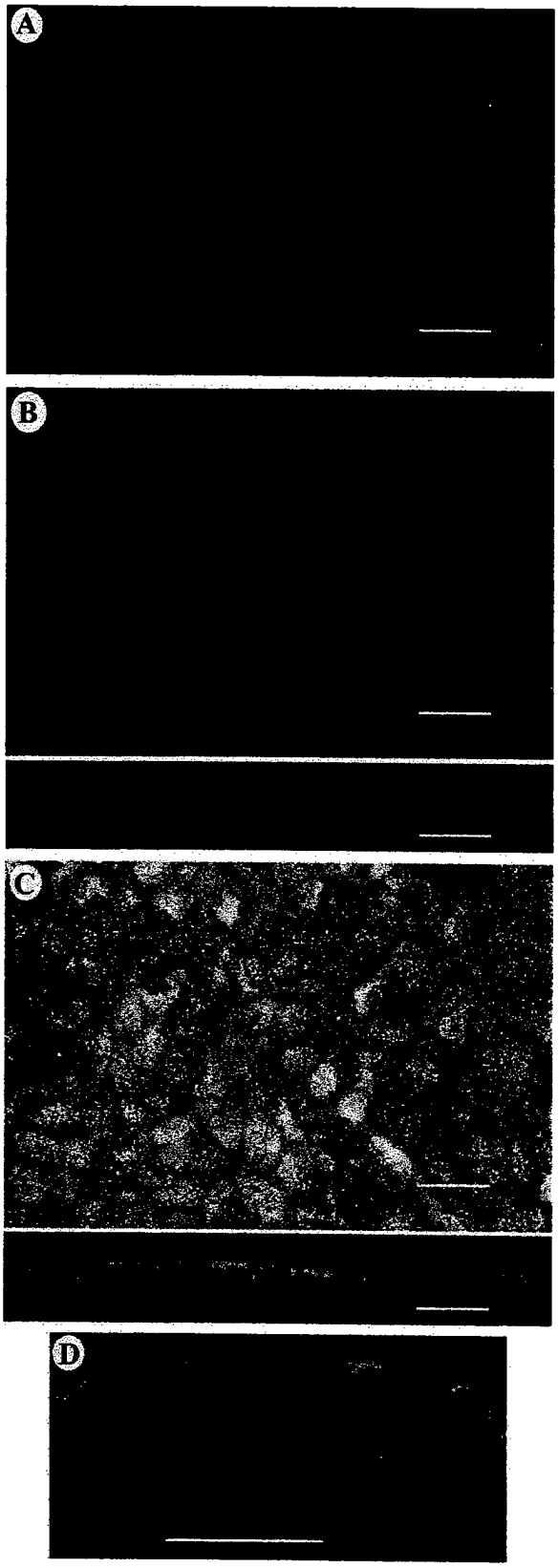

Fig. 4. Immunofluorescent localization of MRP2 in Caco-2 cell monolayers. Expression and localization of MRP1 and MRP2 was examined by indirect immunofluorescence (green signal). Nuclei were detected with propidium iodide (red signal). Scale bar, 10 μm. A and the top of B and C are top-down views of the monolayer (original magnification, 40× water). The bottom of B and C are optical sections perpendicular to the plane of the cell layer. A, confluent Caco-2 monolayer in which primary antibodies were omitted from staining procedure. B, confluent Caco-2 monolayer stained with anti-MRP1 antibodies, MRPm6. C, confluent Caco-2 monolayer stained with anti-MRP2 antibodies, M_2III-6. D, MRP2 localization (M_2III-6) in a 5-μm frozen section cut perpendicular to the plane of the cell monolayer (original magnification, 100× oil).

50 μM MK-571. No absorption was detected in the presence of 50 μM verapamil.

Immunofluorescent Localization of MRP2 in Caco-2 Cell Monolayers. The subcellular distribution of MRP isoforms was visualized in confluent Caco-2 cell monolayers grown on Transwells by indirect immunofluorescent localization with a laser scanning confocal microscope. Cells were seeded onto polycarbonate inserts and grown under conditions identical to those used to grow cells for transport studies and monolayer integrity was determined by TEER. Consistent with our previous data from Western blot analysis (Walle et al., 1999b), monolayers stained with the anti-MRP1 antibody MRPm6 were void of green signal and appeared similar to control samples where primary antibodies were omitted (Fig. 4, A and B). Identical results were obtained with MRPr1 (data not shown). In contrast, samples stained with the anti-MRP2 antibody M_2III-6 demonstrated significant green signal (Fig. 4C) and similar results were observed with M_2I-4 (data not shown). Optical sectioning of the *z*-series perpendicular to the plane of the cell monolayer demonstrated that the subcellular localization of MRP2 was restricted to the apical side of the cells (Fig. 4C, bottom), whereas no evidence for MRP1 was observed (Fig. 4B, bottom). To enhance resolution and verify the apical staining of MRP2, we examined MRP2 localization in 5-μm sections cut perpendicular to the plane of the cell monolayer. Sections stained with M_2III-6 showed that MRP2 immunostaining was primarily confined to the apical plasma membrane, with some diffuse intracellular staining (Fig. 4D).

Discussion

A large body of evidence supports a beneficial role for flavonoids in human health. Although the flavonoid content in the human diet is significant, dietary forms of quercetin have demonstrated very poor and variable absorption in rats and humans (Gugler et al., 1975; Ueno et al., 1983; Hollman et al., 1997; Manach et al., 1997). To understand the mechanisms governing the intestinal absorption of flavonoids we have examined transport of quercetin 4′-β-glucoside with human Caco-2 cell monolayers, an accepted model of the human intestinal epithelium. Previously, we have shown that although quercetin 4′-β-glucoside is not absorbed across this intestinal epithelium, it is effluxed (Walgren et al., 1998). This unidirectional flux suggested the possibility that an efflux pump was involved, and in this study, we have presented evidence to support this hypothesis. First, the transport of quercetin 4′-β-glucoside in the basolateral-to-apical direction demonstrated saturation over the concentration range of 10 to 300 μM, consistent with a saturable secretory mechanism. Second, the efflux of quercetin 4′-β-glucoside was competitively inhibited by MK-571, a selective inhibitor of the closely related MRP1 and MRP2 isoforms (Jedlitschky et al., 1994; Leier et al., 1994; Büchler et al., 1996). This observation in combination with the identification of MRP2 but not MRP1 in our Caco-2 cell monolayers

supports a role for MRP2 in the efflux of quercetin 4′-β-glucoside across the apical membranes of enterocytes.

Although MRP1 was originally associated with chemotherapeutic resistance, MRP isoforms have since been found to be constitutively expressed in a number of tissues. Within the human intestine, Northern blot analysis demonstrates expression of the message for MRP1, MRP2, MRP3, and MRP5 (Kool et al., 1997). Although the spectrum of substrates is similar or identical for MRP1 and MRP2 (Jedlitschky et al., 1994; Leier et al., 1994; Müller et al., 1994), the cellular localization of these two proteins is different. In hepatocytes, MRP1 is present in lateral membranes, whereas MRP2 is restricted to apical canalicular membranes (Büchler et al., 1996; Roelofsen et al., 1999). Similar to MRP1, MRP3 appears to be expressed in the basolateral membrane of human hepatocytes (König et al., 1999). Currently, little is known about the transport characteristics and cellular localization of MRP5.

Although the cellular localization of the MRP isoforms has been determined within the liver, their localization within the gastrointestinal tract is uncertain. Flens et al. (1996) have previously examined MRP1 expression by immunohistochemistry in normal human tissues. Within the small intestine, epithelial cells demonstrated a supranuclear cytoplasmic staining, but the brush border and goblet cells were negative. Despite a recent claim that Caco-2 cells express MRP1 (Gutmann et al., 1999), previous efforts in our laboratory with Western blot analysis have demonstrated the presence of MRP2 but not MRP1 in Caco-2 cells (Walle et al., 1999b). This apparent discrepancy may result from differences in culture conditions because age and seeding density have been shown to influence expression of MRP1 and MRP2 in cultured hepatocytes (Roelofsen et al., 1999). In this study, we have examined confluent Caco-2 cell monolayers for expression of MRP1 and MRP2 by indirect immunofluorescent localization with laser scanning confocal microscopy. Monolayers were grown in Transwells under identical conditions to those used to grow cells for our transport studies. In agreement with our previous finding, we observed expression of MRP2 but not MRP1. The immunostaining of MRP2 was primarily confined to the apical plasma membrane. This pattern of distribution is consistent with the distribution observed in polarized hepatocytes and similar to that observed in MRP2-transfected Madin-Darby canine kidney cell monolayers (Evers et al., 1998). An apical localization of MRP2 within the intestine would place this transporter in a position to both protect against the absorption of compounds from the lumen and secrete agents into the lumen for removal from the body.

Our kinetic studies with intact cell monolayers revealed that the efflux of quercetin 4′-β-glucoside was competitively inhibited by MK-571 with an apparent K_I of 5.6 μM. This value is similar to values observed by Paul et al. (1996) with membrane vesicles from NIH 3T3 cells transfected with MRP1 (K_I = 2.5 μM) but less potent than values reported by Jedlitschky et al. (1994) who used membrane vesicles from MRP1-transfected HeLa cells (K_I = 0.7 μM). Although comparable values from MRP2 membrane vesicles are not available, it has previously been reported that MK-571 is a slightly less potent inhibitor of MRP2 (Büchler et al., 1996).

Inhibition of quercetin 4′-β-glucoside efflux by MK-571 in combination with the identification of MRP2 expression in the apical membranes of Caco-2 cell monolayers strongly implicates a role for MRP2 in the transcellular efflux of this glucoside. The apparent affinity of quercetin 4′-β-glucoside for MRP2 (K_m = 43.6 ± 7.4 μM) does not place this agent among the highest affinity substrates for this transporter. However, the apparent affinity does lie within the predicted lumenal concentration of quercetin 4′-β-glucoside after consumption of a single meal in a typical Western diet (Walgren et al., 1998). This is significant because it allows transport to increase in proportion to increased consumption. A transporter with a lower K_m would already be functioning close to the V_{max} and would therefore be unable to compensate for such an increase in concentration.

The spectrum of agents recognized as substrates by MRP1 and MPR2 is similar, consisting primarily of organic anions, and is principally composed of glutathione and glucuronic acid conjugates of lipophilic compounds (König et al., 1999). It is therefore surprising that quercetin 4′-β-glucoside, an uncharged molecule, is a substrate for MRP2. Previously, in a preliminary study, we have shown evidence that another glucoside, genistin, is a substrate for MRP2 (Walle et al., 1999a). The finding that genistin and quercetin 4′-β-glucoside are both substrates for MRP2 suggests that a large new class of agents may be substrates for MRP-mediated transport.

In agreement with the hypothesis that MRP2 is limiting transcellular absorption of quercetin 4′-β-glucoside we have previously demonstrated a lack of apical-to-basolateral absorption of this agent across Caco-2 monolayers (Walgren et al., 1998). To further test this hypothesis we have examined the transcellular absorption of quercetin 4′-β-glucoside, at various substrate concentrations, in the presence of 50 μM MK-571. In contrast to control treatments that did not demonstrate absorption at any substrate concentration, a modest absorption was observed at 250 μM quercetin 4′-β-glucoside in the presence of 50 μM MK-571, supporting this hypothesis but also suggesting that an additional transport mechanism may be involved, preventing quercetin 4′-β-glucoside from crossing the basolateral membrane. Toxicity of MK-571 at higher concentrations and a limited solubility range for quercetin 4′-β-glucoside have limited further testing (data not shown).

In summary, this study demonstrates that MRP2 is localized to the apical membrane of Caco-2 cells and that this protein both limits the absorption of and mediates the efflux of quercetin 4′-β-glucoside across Caco-2 cell monolayers.

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The Xenobiotic Transporter, MRP2, in Epithelia From Insects, Sharks, and the Human Breast: Implications for Health and Disease

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ABSTRACT A large number of mechanisms, including special excretory transporters, have evolved to help organisms excrete deleterious xenobiotics and endogenous molecules. We have examined the xenobiotic transport function of a putative multidrug resistance associated protein, MRP2, in three different epithelia: the insect renal (Malpighian) tubules, the secretory tubule of the shark rectal gland, and in ductules of the human breast. In the case of the insect and shark, transporter activity occurs in epithelia capable of great fluid transport. In the case of the insect Malpighian tubule, understanding the underlying mechanisms of this transporter may help with efforts to control populations of disease-carrying agriculturally important insects. In striking contrast, ductule architecture in nonlactating human breast ductules is that of an epithelium with a closed lumen. Immunocytochemical studies show that MRP2 is localized in the apical region of the ductule epithelial cells. In this unique case, MRP2 substrates transported into the lumen could possibly be concentrated. Transport substrates of MRP2 include carcinogens as well as antioxidants and other salutary molecules. Thus, in the breast ductule, MRP2 may play a significant role in breast epithelial cell health and cancer carcinogenesis. *J. Exp. Zool. 300A:91–97, 2003.*

INTRODUCTION

Organisms use energy-dependent excretory transport as one mechanism to limit toxic effects of xenobiotics and endogenous molecules (Miller et al., '98). Many of these transporters are members of the ATP-binding-cassette (ABC) superfamily of membrane transporters (reviewed in König et al., '99). Several important transporters in this superfamily are the permeability glycoprotein (PgP), and the multidrug resistance associated proteins, the MRPs. These proteins function as energy-dependent efflux pumps for a variety of structurally diverse chemotherapeutic agents, molecules contained in our diet, and endogenous molecules, including hormones. Pgp and one of the MRPs, MRP2, are expressed in many tissues of the body, especially in polarized epithelial cells of the liver, intestine, and kidney, where they are located on the apical side of the cell. In this apical location, xenobiotic transporters can expel excreted products into ducts or other epithelia-lined tubes that lead directly to the outside world. This review focuses on two members of this ATP superfamily, MRP1, an MRP family member located on the basolateral side of epithelial cells, and MRP2. The substrates of these transporters are generally amphiphilic anions. The parent lipophilic compound is usually transported as a conjugate to glutathione, glucuronate, or sulfate, and transport activity is linked to intercellular metabolism of their substrates. Some molecules are transported without conjugation, sometimes in association with glutathione. Since these transporters export a wide variety of

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chemotherapeutic drugs, most of our knowledge about the substrates of these transporters is derived from study of drugs and drug resistance, and not molecules in the diet. This review focuses our attention on MRP1 and MRP2 substrates derived from the diet.

We will review our research on two model systems from lower organisms, the insect Malpighian tubule and the shark rectal gland secretory tubule. Specifically we have examined the Malpighian tubule of the domestic cricket, *Acheta domesticus* (Karnaky et al., 2000, 2001b) and the secretory tubules of the dogfish shark, *Squalus acanthias*, rectal gland (Miller et al., '98, 2002). We will also review our findings on MRP1 and MRP2 in human breast epithelial cells (Karnaky et al., 2001a).

The Insect Malpighian tubule (MT)

Renal (Malpighian) tubules of insects have served as an important model system to study the neurohumoral control of fluid and ion transport, as well as the excretory mechanisms of certain organic molecules (Dow et al., '94; Gaertner et al., '98). Various species of insects have anywhere from four to over 200 of these gut evaginations. These work in concert with the intestine to effect the excretion of huge quantities of fluid, as in the case of the mosquito after the animal bites. The relative ease with which these tubules can be isolated and utilized individually or in groups to measure transport of salts or organic molecules makes them especially attractive models (Dow et al., '94). Microscopic, isotopic, chromatographic, and fluorescent methods have been used in these studies. For example, physiological studies by Gaertner et al. ('98) have shown that transport of nicotine and vinblastine in MT of the tobacco hornworm is inhibitable by verapamil. This finding suggests the presence of a Pgp-like transport system. Isolated tubules can be viewed with a confocal fluorescent microscope after exposure to various substrate or transport inhibitors. The fluorescence intensities can be measured from stored images using appropriate software. The transport of the MRP2 substrate, Texas Red (sulforhodamine 101: TR), into the lumens of isolated *A. domesticus* MT is so robust that it can also be viewed with dissecting and light microscopes (Karnaky et al., 2001b). Potassium cyanide (KCN) inhibited luminal accumulation significantly; this demonstrates the ATP dependence of this transport process. The nonfluorescent MRP2 substrate, chlorodinitrobenzene, also inhibited the transport. Immunocytochemical studies showed that luminal cells were stained with an antibody to a sequence of rat MRP2, further suggesting that a MRP2–like transporter is present in MT. Similar results were obtained from the study of the common American cockroach, *Periplaneta america* (Karnaky et al., 2000).

These reports are some of the first to document the transport of an MRP2 substrate by the insect MT and one of the first applications of the confocal fluorescent microscope to document the transport of organic compounds into these tubules. This model system presents some unique advantages for the study of MRP2 transport mechanisms. First, the system can be used to collect excreted MRP2 substrates under carefully controlled in vitro conditions. With a gut cannula it is possible to collect an analyzable quantity of excretions from all 114 tubules (about 4 microliters/hour) in the cricket (Spring, '87). The robust transport detectable with the dissecting and light microscopes is especially interesting and may allow the development of rapid screening methods to test large numbers of potential MRP2 substrates. Finally, the complete genome is now available for the fly (Boutros and Perrimon, 2000) and should facilitate cloning the MRP2 transporter in the cricket.

MRP2–like transport of xenobiotics in the dogfish shark rectal gland

At the time we first examined the shark rectal gland for the presence of xenobiotic transporters there was a small but growing literature that suggested they were present in aquatic animals (Miller et al., '98, 2002), but nothing was known about the presence of these transporters in elasmobranchs. We therefore examined the elasmobranch rectal gland, which is specialized for salt excretion, and has constituted an excellent model for the study of NaCl secretion (Forrest, '96). The gland consists of numerous blind-ended secretory tubules that converge into a central duct that leads to the intestine. Until our studies, only NaCl and fluid secretion had been described for this gland.

We now briefly describe our findings on the xenobiotic transporters, Pgp and MRP2, in the shark rectal gland (Miller et al., '98, 2002). Isolated secretory tubules were incubated in various fluorescent substrates of Pgp and MRP2 and examined with the confocal fluorescent

microscope. We observed luminal accumulation of TR and fluoroscein methotrexate, two MRP2 substrates. This accumulation was concentrative, saturable, and inhibited by a number of molecules that inhibit MRP2 transport, e.g., cyclosporin A (CSA), chlorodinitrobenzene, leukotriene C_4, and KCN. We also attempted to affect TR transport with probenecid and *P*-aminohippurate, inhibitors of renal organic anion transport, with tetraethylammonium, an inhibitor of organic cation transport and with verapamil, an inhibitor of the P-glycoprotein. None had an effect. TR entered the cells, but its accumulation was neither concentrative, saturable, nor inhibitable. The rectal gland tubules did not secrete the fluorescent organic anion, fluorescein, or fluorescent substrates for the renal organic cation transport system (daunomycin) and the Pgp (daunomycin and NBDL-CSA). An antibody to rat MRP2 was found by immunochemistry to be localized on the luminal membrane of the secretory tubule epithelial cells. The rectal gland secretory tubule of the dogfish shark thus appears capable of actively excreting xenobiotics, with their transport mediated by a shark analog of MRP2, but not by Pgp.

Next we explored the regulation of MRP2 function in shark rectal gland secretory tubules. MRP2-mediated transport in teleost renal proximal tubules was known to be regulated by endothelin (ET), which acts through an ET_B receptor and protein kinase C (Masereeuw et al., 2000). In fish the basolateral ET receptors of the proximal tubules bound endothelin. Activation of protein kinase C decreased the transport of MRP substrates to the lumen. Sharks are known to produce ET and to express ET receptors that are pharmacologically similar to those of mammals (Evans et al., '96). A parallel story was found in the shark rectal gland: ET regulation through an ET_B receptor and protein kinase C (Miller et al., 2002).

Again we used isolated, intact shark rectal gland secretory tubules, the MRP2 substrate, TR, and confocal fluorescence microscopy (Miller et al., 2002). Within minutes, small concentrations of ET, from subnanomolar to nanomolar, reduced the cell-to-lumen transport of this MRP2 substrate. We were able to prevent these effects by a receptor antagonist to ET_B but not by an antagonist to ET_A. An antibody to mammalian ET_B receptors specifically stained the basolateral membrane. Protein kinase C (PKC), but not protein kinase A (PKA), seems to mediate this regulation because a specific, PKC-selective, inhibitor blocked this ET-1 effect, but PKA-selective inhibitors did not.

The implications for endothelin regulation of MRP2 transport are not fully understood. Intestinal epithelial cells are known to possess MRP2 (Fromm et al., 2000) and endothelin B receptors (Egidy et al., 2000). Endothelin regulation of MRP2 may exacerbate the acute inflammation that is encountered in ulcerative colitis. Endothelin secreted by cells in inflamed connective tissue may inhibit MRP2 transport of toxic molecules and thus increase the toxic load in the intestinal epithelial cells.

Role of MRP1 and MRP2 transporters in breast epithelial cell health and carcinogenesis

The last part of this review examines the potential role MRP1 and MRP2 play in influencing the level of exposure of breast tissue to potential environmental determinants. Although these transporters have been studied mainly for their role in liver function and in multidrug resistance in cancer (König et al., '99), there is growing awareness that their substrates also include xenobiotic, carcinogenic, and protective antioxidant molecules (Walle et al., '99a, b).

For as yet unknown reasons, breast cancer incidence rates have been 4-7 times higher in the United States than in China or Japan (Ziegler et al., '93). When Chinese, Japanese, or Filipino women immigrate to the United States, breast cancer risk rises over several generations and approaches that encountered in U.S. whites (Ziegler et al., '93). This suggests that there exist environmental and lifestyle determinants of the disease that are potentially modifiable. But what are these dietary, environmental, and estrogen influences on breast cancer?

Epidemiological and experimental evidence indicates that dietary factors influence the incidence of mammary gland cancer, yet the dietary factors that may cause or contribute to this cancer remain largely unknown (Snyderwine, '94). Sex hormones and reproductive history are important factors in breast cancer. Endogenous and exogenous hormones, such as estrogen, drive cell proliferation; this also increases the accumulation of random genetic errors (Henderson and Feigelson, 2000).

Because estrogen plays a key role in breast cancer, there is intense interest in the role of

phytoestrogens, particularly the isoflavones (Bingham et al., '98), in disease development and prevention. Phytoestrogens present in soy act as partial estrogen agonists or antagonists and can inhibit breast cancer cell proliferation. One of the phytoestrogens present in soy is the isoflavone, genistein, a weak estrogen agonist. Dietary soy supplementation raises levels of this molecule in serum and nipple aspirate fluids (Hargreaves et al., '99). Fritz et al. ('98) hypothesized that early exposure to genistein could provide a lasting protective effect against breast cancer, because dietary intake of genistein brought about a dose-dependent protection against development of chemically-induced mammary tumors in rats.

The mammary gland is the most unusual exocrine gland of the body: in the nonlactating state molecules secreted into the lumen will not be flushed out, as would be the case in the lactating gland. Theoretically, molecules secreted into the lumen could diffuse back across the apical membrane into the lining cells. Transport of molecules into the small luminal volume of the breast ductules could produce significantly high concentrations of those molecules. If transporters were present in the breast ductule epithelial cells and transported at rates like those seen for the Pgp in fish kidney cells (Sussman-Turner and Renfro, '95), the lumen of a 1 mm ductule could harbor 1 nM of the transported molecule in only 10 seconds. With the aid of immunocytochemical and Western blot methods we have localized two membrane xenobiotic transporters, MRP1 and MRP2, in breast ductule and alveolar epithelial cells (Karnaky et al., 2001a). As mentioned above, MRP2 is located at the apical membrane of epithelial cells and excretes anionic hydrophobic substrates, such as 17 beta-estrogen glucuronide, antioxidants, and toxic molecules into the lumens of the kidney, liver, and gut. In isolated and cultured breast ductules, MRP2 is located predominantly in the apical region of luminal epithelial cells. Therefore its action could lead to high concentrations of toxic substrates in sealed, non-flushed lumens. In cultured Caco2 cells, MRP2 is localized at the apical membrane only (Walgren et al., 2000) and in a Western blot it is detected as bands at 60 and 190 kDa. In breast tissue cultures, on the other hand, MRP2 is detected only at 60 kDa. As noted above, MRP1 transports anionic hydrophobic molecules across the basolateral pole of epithelial cells into the connective tissue. In isolated and tissue cultured breast ductules, MRP1 is detected as a band at 190 kDa and is localized in the cytoplasm, but more towards the basal side of the cells, as is typical for numerous epithelial cells (Karnaky et al., 2001). Thus, net toxin concentration in breast ductule lumens may be the result of the difference in transport activity of MPR1, and MPR2. Sandusky et al. (2002) found that 25 of 49 breast carcinomas exhibited a positive response to antibodies to MRP2. Alcorn et al. (2002) have detected MRP1 and MRP2 in epithelial cells isolated from breast milk with the aid of PCR. Figure 1 presents a general model for the role of MRP1 and MRP2 in breast epithelial cells.

As shown in Table 1, all three secretions of breast epithelial cells, breast cyst fluid, nipple aspirate fluid, and breast milk, contain known or potential substrates of MRP1 and MRP2. Table 2 lists MRP1 and MRP2 substrates of potential importance for breast health. The heterocyclic amine, 2-amino-1-methyl-6-phenylimidazo[4,5-b] pyridine (PhIP), which is a breast cancer carcinogen in rats (Ito et al., '91), is formed from its precursors, creatinine, amino acids, and sugars, when meat or fish are cooked. Of the many potential steroid substrates, MRP2 has, as yet, been shown to transport only 17-beta estradiol glucuronide (Loe et al., '96).

As shown in Table 2, MRP1 and MRP2 substrates may be divided into three categories: those likely to be mutagenic or carcinogenic; those that, because of their antioxidant or other properties, may be protective; and estrogen, a major factor in breast cancer. It seems likely, though as yet unproven, that luminally-located MRP2 can transport these substrates to the lumen of the breast ductules. It is also likely that basolaterally-located MRP1 plays an important role by quickly transporting molecules back to the underlying connective tissue.

Outlook

In the last part of this review we have presented suggestive evidence that breast cancer is related to diet and that the two membrane transporters, MRP1 and MRP2, could significantly modulate exposure of breast epithelia to xenobiotic and endogenous molecules. Future studies should seek to show which of these molecules are actually transported and determine whether they affect epithelial function and health. Available information is scant on the normal polarized epithelial cells of the breast and on the transport processes in these cells. It is hoped future research will

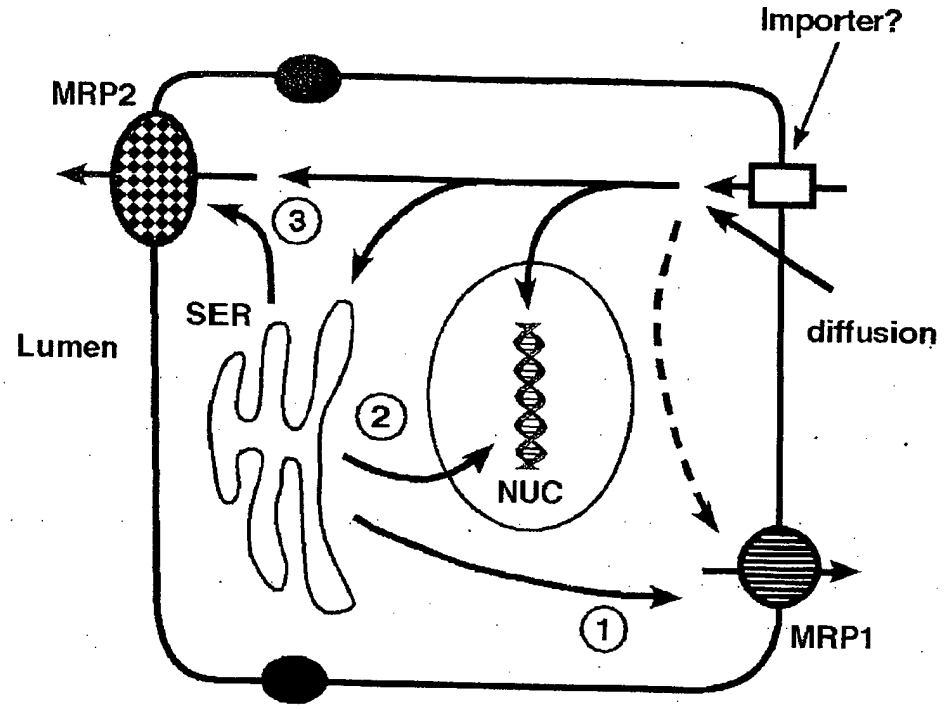

Fig. 1. Potential roles for MRP1 and MRP2 in the transport of xenobiotic and endogenous molecules in breast epithelial cells. In this model, potential substrates would either diffuse into the cell via the basolateral pole or be actively transported by importers yet to be identified. Some of these molecules would be removed from the cell by basolateral MRP1 before they are metabolized (stippled pathway). Other molecules might diffuse into the nucleus (NUC) and cause mutations or protect the DNA. Still other molecules might diffuse to the smooth endoplasmic reticulum (SER) where P450 and transferases are present. After oxidation and conjugation, molecules could take one of three different pathways: 1) back to the basal cytoplasm, where they would be removed from the cell via MRP1; 2) to the nucleus where they might cause mutations; and 3) to the apical MRP2, where they would be transported to the lumen and be concentrated.

TABLE 1. Known and potential substrates of MRP1 and MRP2 in the three fluids produced by human breast epithelia

Fluid	Substrate	Reference
Cyst	estradiol-3-sulfate	(Umaraju et al.,'77)
	estradiol-16-glucosiduronate	
	estradiol-3-sulfate	(Rao et al.,'94)
	dehydroepiandrosterone sulphate	(Boccardo et al.,'88)
	estriol-3-sulfate, Estriol-16-glucosiduronate,	(Miller et al.,'83)
	estriol-3-sulfate-16-glucosiduronate	
	oestrone sulphate	(Hawkins et al.,'85)
	estriol-3-sulfate	(Raju et al.,'85)
Nipple	estrogen	(Petrakis et al.,'87)
aspirate	estradiol (5-45 times higher than serum levels)	
	genistein	(Hargreaves et al.,'99)
Breast milk	ochratoxin A	(Jonsyn et al.,'95; Micco et al.,'95)
	aflatoxin	(Saad et al.,'95)
	PhIP*, PhIP-4'-sulfate	(DeBruin et al., 2001)
	N-2-hydroxy-PhIP-N3-glucuronide	
	over 50 steroids,	(Sahlberg and Axelson,'86)
	80% as sulfates, 15% as glucuronides,	
	5% unconjugated	
	genistein	(Franke et al.,'98)

*PhIP=2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine

TABLE 2. MRP1 and MRP2 substrates of potential relevance to breast ductule epithelial cell health and carcinogenesis

Transporter	Substrate	Cell or Epithelial Model Tested	Reference
	MUTAGENS, CARCINOGENS		
MRP1[a]	aflatoxin B1	Note 1	(Loe et al.,'97)
MRP1	ochratoxin A	HEK-MRP2 cells	(Leier et al., 2000)
MRP2[b]	ochratoxin		(Leier et al., 2000)
MRP2	PhIP[c]	Caco-2	(Walle and Walle,'99)
	ANTIOXIDANTS		
MRP2	chrysin	Caco-2	(Walle et al.,'99b)
MRP2	quercetin-4'-β-glucoside	Caco-2	(Walgren et al.,'98)
MRP2	genistein		(Jager et al.,'97; Walle et al.,'99a)
MRP2	glutathione STEROID		(König et al.,'99)
MRP1	17 beta estradiol-glucuronide		(Loe et al.,'96)

[a]MRP1=multidrug resistance associated protein 1; [b]MPR2=multidrug resistance associated protein 2; Note 1=plasma membrane vesicles from MRP-transfected HeLa cells; [c]PhIP=2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine.

investigate this area of epithelial physiology. Such studies should help determine whether and how breast epithelial cells respond to dietary molecules and could lead to specific dietary recommendations with profound consequences for human health.

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