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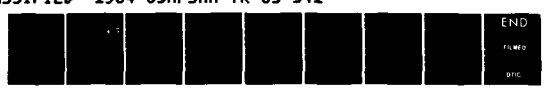

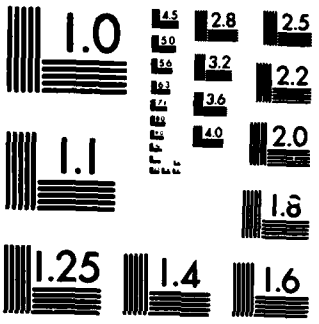

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The thermal induction of chemiluminescence of luminol-horseradish peroxidase-labeled erythrocytes from pigs, rats, and man was studied. The luminescent responses of rat, porcine, and human erythrocytes to heating were linear in respect to logs of counts per minute versus temperature. Landrace-Duroc crossbred pigs with a history of malignant hyperthermia (porcine stress syndrome) and Poland-China-miniature pigs inbred for malignant hyperthermia (MH) yielded erythrocytes with high-level thermochemiluminescence (TCL). Sprague-Dawley rat erythrocytes were intermediate in their production of TCL. Normal human and MH-resistant miniature swine erythrocytes produced low-level TCL. However, pretreatment of human erythrocytes with 1-chloro-2,4-dinitrobenzene (CDNB) resulted in high level TCL. Furthermore, halothane enhanced the TCL of CDNB-treated human erythrocytes and Landrace-Duroc porcine erythrocytes that were not treated with CDNB. Red blood cells from pigs susceptible to the porcine stress syndrome demonstrated a TCL response very similar to CDNB-treated erythrocytes.

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ANALYTICAL BIOCHEMISTRY 143, 231–236 (1984)

Thermochemiluminescent Assay of Porcine, Rat, and Human Erythrocytes for Antioxidative Deficiencies

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The thermal induction of chemiluminescence of luminol–horseradish peroxidase-labeled erythrocytes from pigs, rats, and man was studied. The luminescent responses of rat, porcine, and human erythrocytes to heating were linear in respect to logs of counts per minute versus temperature. Landrace–Duroc crossbred pigs with a history of malignant hyperthermia (porcine stress syndrome) and Poland–China–miniature pigs inbred for malignant hyperthermia (MH) yielded erythrocytes with high-level thermochemiluminescence (TCL). Sprague–Dawley rat erythrocytes were intermediate in their production of TCL. Normal human and MH-resistant miniature swine erythrocytes produced low-level TCL. However, pretreatment of human erythrocytes with 1-chloro-2,4-dinitrobenzene (CDNB) resulted in high-level TCL. Furthermor halothane enhanced the TCL of CDNB-treated human erythrocytes and Landrace–Duroc porcine erythrocytes that were not treated with CDNB. Red blood cells from pigs susceptible to the porcine stress syndrome demonstrated a TCL response very similar to CDNB-treated erythrocytes.

KEY WORDS: erythrocytes; thermochemiluminescence; peroxidase; hemoglobin; autoxidation; luminol.

Oxyhemoglobin undergoes spontaneous autoxidation in erythrocytes, converting 3% of cellular hemoglobin to methemoglobin daily (1). In this process, superoxide and subsequently hydrogen peroxide and lipid peroxides are generated (1). The addition of various anionic nucleophiles (i.e., azide, halides, and thiocyanate) accelerates the autoxidation reaction (2). Furthermore, increasing the environmental temperature by 3°C doubles the rate of oxyhemoglobin autoxidation under physiological conditions (3). The autoxidation is minimized in erythrocytes by various endogenous enzymes such as superoxide dismutase, catalase, and glutathione peroxidase (4). However, the inhibition or inherited deficiency of these enzymes or deficiencies in key coenzymes may lead to increased susceptibility to oxyhemoglobin autoxidation (1,5).

We report the thermal induction of chemiluminescence of luminol–horseradish peroxidase-labeled erythrocytes from pigs, rats, and man. We also examined the association of this luminescence with malignant hyperthermia (MH)[1] in pigs and with 1-chloro-2,4-dinitrobenzene treatment of human erythrocytes.

MATERIALS AND METHODS

Specimens. Red blood cells (RBCs) were obtained from Landrace–Duroc crossbred pigs with a herd history of MH, Poland–China–miniature crossbred pigs inbred for MH, miniature swine resistant to MH, Sprague–Dawley rats, and a normal human. The number of cells per milliliter was determined with a Model 2B1-6 Coulter counter. Freshly

[1] Abbreviations used: MH, malignant hyperthermia; RBC, red blood cell; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; BSA, bovine serum albumin; TCL, thermochemiluminescence; CDNB, 1-chloro-2,4-dinitrobenzene.

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drawn RBCs (containing sodium citrate as an anticoagulant) were prepared for labeling by washing them free of plasma and suspending them in pH 6.9 phosphate-buffered saline (PBS, 0.1 M sodium phosphate buffer with 0.154 M NaCl) containing 0.5% (v/v) glutaraldehyde. The tanning with glutaraldehyde proceeded for 2 h at 4°C. The cells were washed free of unbound glutaraldehyde, centrifuged at 600*g*, and resuspended in pH 6.9 PBS containing 1 mg/ml horseradish peroxidase (HRP) and 1 mg/ml bovine serum albumin (BSA) with noncovalently bound luminol (5-amino-2,3-dihydrophthalazine-1,4-dione) (6). This preparation was incubated overnight at 4°C. Finally, the cells were washed and stored in pH 7.4 PBS containing 0.5% D-glucose.

Thermochemiluminescence. To generate chemiluminescence, the cells were heated in a water bath to the desired temperature ±0.2°C within the range of 30 to 55°C and immediately counted in a Beckman LS230 liquid scintillation counter set in the out-of-coincidence mode. The quantities of RBCs used are described in the figure legends. Inhibition studies were performed by adding superoxide dismutase, catalase, copper sulfate, or sodium nitrite in quantities described in Table 1 to a 1-ml cell suspension of labeled rat RBCs (1×10^8 cells) in pH 7.4 PBS. The enzymes remained in solution during the thermochemiluminescent (TCL) assay. The cells were washed free of copper sulfate or sodium nitrite by centrifugation and resuspension after 30 min of preincubation prior to the TCL assay. In these inhibition studies, the cells were heated to 50°C before chemiluminescence was measured.

1-Chloro-2,4-dinitrobenzene treatment. Some samples of human RBCs were pretreated with 1-chloro-2,4-dinitrobenzene (CDNB) prior to luminescent labeling to conjugate in excess of 80% of the endogenous reduced glutathione as described by Awasthi *et al.* (7). This treatment was accomplished by incubating the cells in pH 7.4 PBS containing 0.5 mM CDNB in a water bath for 15 min at 37°C. The cells were then washed and resuspended in fresh pH 6.9 PBS for labeling of cells as previously described.

Halothane effects. To test the effects of the metabolic stressor halothane (2-bromo-2-chloro-1:1:1-trifluorethane) on TCL, 20 μl of halothane were added to 1 ml of either porcine or human RBCs prior to induction of TCL. The scintillation vials were sealed to prevent escape of the volatile halothane during heating and counting.

RESULTS

The luminescent responses of luminol-labeled rat, porcine, and human RBCs to heating are linear in respect to logs of counts per minute (cpm) versus temperature (Fig. 1). There was a significant difference between the responses of MH-sensitive Landrace-

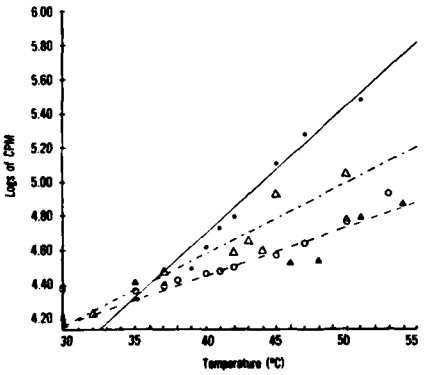

FIG. 1. Thermally induced chemiluminescence of luminol–horseradish peroxidase-labeled erythrocytes of various species. The red blood cells (1×10^9 of each type) were labeled with luminol and horseradish peroxidase (HRP) and heated to induce chemiluminescence as described under Materials and Methods. Each point represents the mean of three samples. The standard error of the instrument was ±2%. Closed circles represent Landrace–Duroc crossbred porcine cells; open circles, miniature porcine cells; open triangles, Sprague–Dawley rat cells; closed triangles, human RBCs. The solid line is the linear regression for Landrace–Duroc data (correlation coefficient $r = 0.979$); long and short dashed line, rat data regression ($r = 0.940$); single dashed line, regression for combined human and miniature swine data ($r = 0.959$, pooled since they were statistically indistinguishable).

TABLE 1

THERMALLY INDUCED (50°C) CHEMILUMINESCENCE OF (1×10^8) LUMINOL-HORSERADISH PEROXIDASE-LABELED RAT ERYTHROCYTES

Reagent	cpm ± SD[a] ($N = 3$)	Percentage activity
None	146,519 ± 2777	100
Superoxide dismutase (5 μg)	49,476 ± 6958	33.8
Catalase (60 μg)	46,566 ± 9123	31.28
$CuSO_4$ (9 μmol)	34,145 ± 2691	23.3
$NaNO_2$ (33 μmol)	31,182 ± 2983	21.3

[a] The empty vial cpm were 17,905 ± 4662 ($N = 13$).

Duroc crossbred pig and MH-resistant miniature pig RBCs. However, normal human and MH-resistant miniature pigs showed the same relative response as a function of temperature. Sprague-Dawley rat RBCs were intermediate in their sensitivity to heating.

Treatment of labeled rat RBCs with superoxide dismutase or catalase during heating the cells to 50°C resulted in inhibition of the luminescence (Table 1). These responses indicate the participation of superoxide and hydrogen peroxide in the chemiluminescent reaction. Copper sulfate and sodium nitrite which convert oxyhemoglobin to methemoglobin also inhibited the luminescence. The formation of methemoglobin in RBCs treated with copper sulfate was grossly evident by their red-brown color.

Upon storage of labeled Landrace-Duroc porcine erythrocytes for a week, the cells declined in TCL. TCL of 1×10^9 labeled RBCs from a Landrace-Duroc pig (not described in Fig. 1) declined in luminescence from 310,000 cpm at 45°C on the first day after labeling to 206,200 cpm at 45°C on the seventh day after labeling. This decline was apparently from methemoglobin formation, since the samples slowly developed the color changes seen with copper sulfate.

When the labeled Landrace-Duroc RBCs were treated with halothane the TCL was greatly enhanced (Fig. 2). RBCs (3×10^8 cells) from inbred Poland-China-miniature pigs with malignant hyperthermia also showed a high level of TCL (Fig. 4).

Normal human RBCs not ony displayed

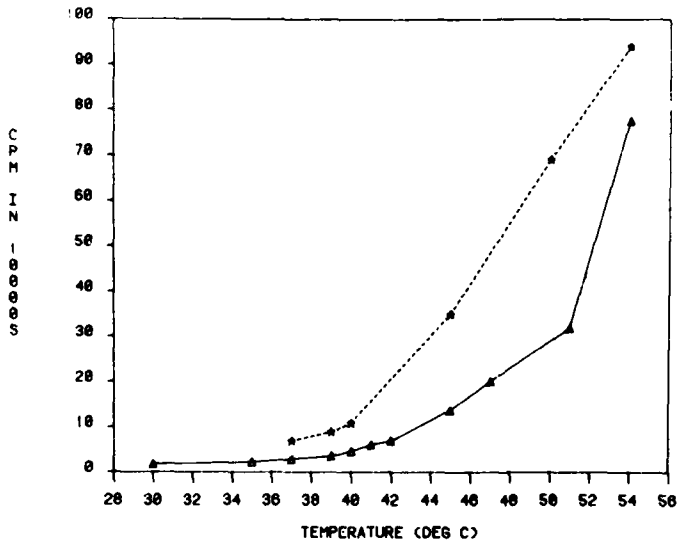

FIG. 2. Effect of halothane on thermochemiluminescence of luminol-labeled Landrace-Duroc erythrocytes (1×10^9 cells). The triangles and solid line represent the response without halothane; the stars and dashed line represent the response with halothane.

low-level TCL but also failed to show the enhancement of TCL with halothane (Fig. 3). Only after treatment with CDNB did these cells demonstrate TCL rivaling that of the Landrace–Duroc or Poland–China–miniature porcine RBCs. This showed that the fixing and labeling process in itself did not result in TCL.

To confirm the presence of luminol following the TCL assays (in all cases), each milliliter of cells was treated with 100 μl of 0.1 N sodium hydroxide. This treatment induced luminescence in excess of one million cpm. Therefore, luminescent material was present at the surfaces of all labeled cells examined, even after treatment with copper sulfate, sodium nitrite, or brief inductions of luminescence by heating. The NaOH-induced counting rates also indicated that the luminol is present in excess. This suggests that the TCL assay is a valid measure of autoxidation kinetics.

To control for luminescence originating from the reagents independent of the cells, 1 mg HRP/ml and 1 mg BSA with luminol/ml of pH 7.4 PBS were heated and observed. Figure 4 shows that the TCL of these reagents is at background (see footnote to Table 1). Furthermore, Poland–China–miniature porcine RBCs coated with glutaraldehyde alone or glutaraldehyde, HRP, and BSA (without luminol) produced only low-level TCL (Fig. 4). These controls indicated that luminol was the principal source of TCL and the oxidizing agent which initiated the TCL originated in the RBCs. Figure 4 shows that MH-sensitive Poland–China–miniature porcine RBCs, produce a high level of TCL.

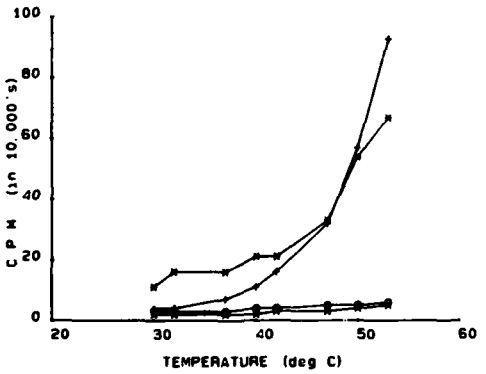

FIG. 3. Thermochemiluminescence of luminol–horseradish peroxidase-labeled human RBCs (3×10^8 cells). X's represent values of normal human RBCs; open circles, normal RBCs exposed to halothane; crosses, 1-chloro-2,4-dinitrobenzene (0.5 mM)-treated cells; stars, 1-chloro-2,4-dinitrobenzene-treated cells exposed to halothane. All values are means of triplicate values.

DISCUSSION

The erythrocytic metabolic pathways are integrated for regeneration of reduced hemoglobin and disposal of superoxide (O_2^-) and peroxides produced by oxyhemoglobin autoxidation (Fig. 5). The dissociation of oxyhemoglobin into methemoglobin and superoxide has a heat of activation of 32 kcal/mol under physiological conditions (3). However, even in the presence of elevated temperature superoxide and peroxide may not be observed because of scavenging by superoxide dismutase, catalase, and glutathione peroxidase as shown in Fig. 5.

The tremendous increase in TCL by pretreatment of human RBCs with CDNB suggests a significant role of glutathione and subsequently glutathione peroxidase in scavenging oxidizing agents produced by heated RBCs. CDNB is irreversibly conjugated with glutathione in RBCs by the action of glutathione-*S*-transferase (7). The thermochemiluminescent assay as depicted in Fig. 6 requires the escape of these oxidizing agents to the outer cell membrane surface. Evidently the structure of RBCs allows for the escape of either some superoxide or peroxide before it can be scavenged (Table 1). Anionic channels in RBCs which allow the transmembrane passage of superoxide have been reported (8). The enhancement of TCL by halothane in human RBCs treated with CDNB is consistent with the observations of Schanus *et al.* (5). They showed that glutathione peroxidase

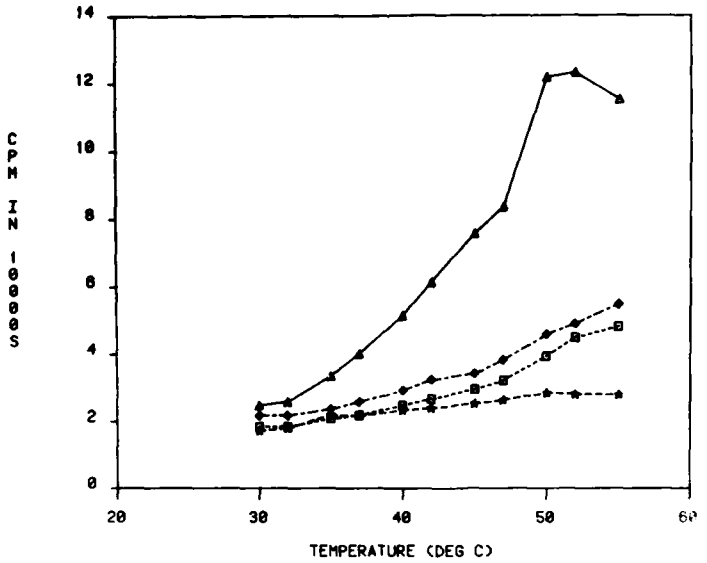

FIG. 4. Thermochemiluminescence of RBCs from Poland–China–miniature pigs. The triangles represent 3×10^8 cells labeled with luminol, bovine serum albumin (BSA), and HRP; diamonds, 3×10^8 cells coated with BSA and HRP; squares, 3×10^8 cells tanned with glutaraldehyde; stars, 1 ml of 1 mg HRP/ml and 1 mg BSA with noncovalently linked luminol/ml in pH 7.4 PBS. Each point represents the mean of 3 data points per day for 3 separate days ($N = 9$). The mean standard deviation for luminol-labeled RBCs was ±12.1% with a range of 9.3 to 17.1%; for cells treated with glutaraldehyde only, SD = ±10.6%, range: 6.6 to 20.4%; for BSA- and HRP-coated cells, SD = ±14.7%, range: 10.8 to 18.7%; for reagents only, SD = ±10.9%, range: 6.9 to 14.5%.

activity deficiency leads to increased RBC peroxidation and increased susceptibility of the cells to halothane stress.

Malignant hyperthermia has been associated with inherent glutathione peroxidase deficiency in swine (5). This deficiency allows accumulation of hydroperoxides generated by oxidizing agents. Our assay both initiates

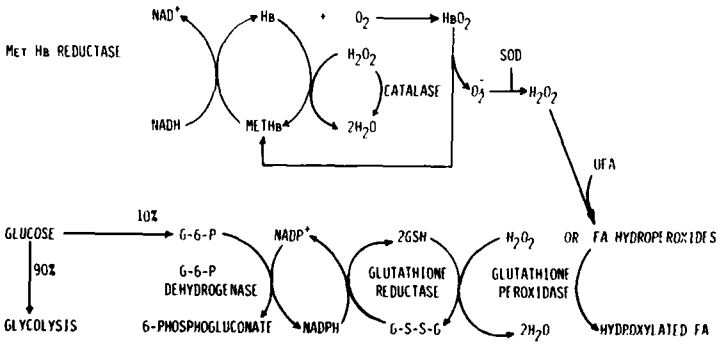

FIG. 5. Metabolic pathways which control autoxidation of the red blood cells. Hb = hemoglobin; HbO_2 = oxyhemoglobin; MetHb = methemoglobin; SOD = superoxide dismutase; GSH = reduced glutathione; G-S-S-G = oxidized glutathione; G-6-P = glucose 6-phosphate; UFA = unsaturated fatty acids.

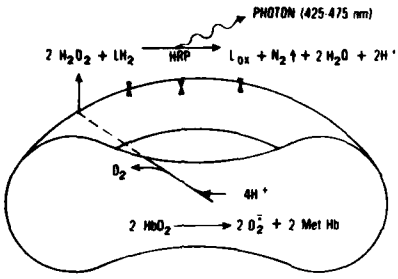

FIG. 6. Schematic of the autoxidation of a red blood cell measured by luminol chemiluminescence. LH_2 = luminol; L_{ox} = oxidized luminol (aminophthalic acid).

the production of peroxides and detects peroxides in RBCs.

Our data from MH-sensitive Landrace–Duroc and Poland–China–miniature pigs and MH-resistant miniature pigs indicates that the TCL assay may have value as a clinical assay for malignant hyperthermia. Furthermore, our results with normal and CDNB-treated human RBCs suggest that deficiencies in human glutathione peroxidase activity may be detected by the TCL assay.

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