Heat Shock Protein-70 Inducers and iNOS Inhibitors as Therapeutics to Ameliorate Hemorrhagic Shock

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ABSTRACT

Hemorrhagic shock is the principal cause of death of soldiers in the battlefield. Although the underlying mechanisms are still not fully understood, it has been shown that nitric oxide (NO) overproduction and inducible nitric oxide synthase (iNOS) overexpression play important roles in producing injury caused by hemorrhagic shock. In addition, polymorphonuclear neutrophils (PMN) infiltrate injured tissues and leukotriene B_4 (LTB_4) generation increases. In a hemorrhage/resuscitation-induced injury model, iNOS, cyclooxygenase-2, and CD14 are all upregulated. The early response genes such as iNOS and cyclooxygenase-2 then promote the inflammatory response by the rapid and excessive production of NO and prostaglandins, respectively. It has been evident that either upregulation of inducible heat shock protein 70 kDa (HSP-72) or downregulation of iNOS can limit tissue injury caused by ischemia/reperfusion or hemorrhage/resuscitation. In our laboratory, geldanamycin, a member of ansamycin family, induces HSP-70i overexpression, to inhibit iNOS expression, to reduce cellular caspase-3 activity, and to preserve cellular ATP levels. With future combat operations expecting longer evacuation times and limited availability of medical supplies far-forward, significant improvements in fluid resuscitation will be required if potentially salvageable injured are to be saved. The action of adjunct therapy agents in the resuscitation fluid that limit tissue injury will further reduce the hemorrhage-induced injury. Since HSP-70i overexpression and iNOS inhibition exhibit cytoprotection, a compound such as geldanamycin as a HSP-70i inducer as well as an iNOS inhibitor will represent a best additive to resuscitation fluids that may reduce hemorrhage-induced injury.

1.0 INTRODUCTION

Hemorrhagic shock is the leading cause of death and complications in combat casualties and civilian trauma. It has been shown to cause systemic inflammatory response syndrome, multiple organ dysfunction, and multiple organ failure [Baue 1998]. Analysis of the historical data (Table 1) demonstrates that the mortality rates from World War I, World War II, the Korean War, and the Vietnam Conflict are very similar and also show no improvement over this period. Moreover, the rates of soldiers who died of wounds after reaching a treatment facility during the Vietnam Conflict did not improve in spite of the rapid evacuation times. The published data also show that approximately half of those killed in action died of hemorrhagic shock. Hemorrhagic shock is an old problem and to find useful remedies that are capable of reducing casualty caused by hemorrhagic shock is a most important task in combat medicine.

Paper presented at the RTO HFM Symposium on "Combat Casualty Care in Ground Based Tactical Situations: Trauma Technology and Emergency Medical Procedures", held in St. Pete Beach, USA, 16-18 August 2004, and published in RTO-MP-HFM-109.

Report Documentation Page

Form Approved
OMB No. 0704-0188

Public reporting burden for the collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to a penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number.

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 SEP 2004	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
Heat Shock Protein-70 Inducers and iNOS Inhibitors as Therapeutics to Ameliorate Hemorrhagic Shock	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, U.S.A.; Departments of 2Medicine and of 3Pharmacology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799, U.S.A.;	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

See also ADM001795, Combat Casualty Care in Ground-Based Tactical Situations: Trauma Technology and Emergency Medical Procedures (Soins aux blessés au combat dans des situations tactiques : echnologies des traumas et procédures médicales durgence)., The original document contains color images.

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**12**	

Table 1: Historical Data of Soldiers Killed in Action and Died of Wounds [Rhee 2003]

War	Killed in Action (%)	Died of Wounds (%)
World War I	19.6	8.1
World War II	19.8	3.0
Korean War	19.5	2.4
Vietnam Conflict	20.2	3.5

In this paper, we will discuss effects of hemorrhagic shock, resuscitation fluids used for hemorrhagic shock, and amelioration of additives to resuscitation fluids. The additives will be focused on inducers of heat shock protein 72 kDa (HSP-72, an inducible form of the HSP-70 family) and inhibitors of inducible nitric oxide synthase (iNOS).

2.0 EFFECTS OF HEMORRHAGIC SHOCK

Hemorrhage leads to systemic inflammatory response syndrome, multiple organ dysfunction, and multiple organ failure [Baue 1998]. A variety of biomolecules is known to be involved in this response. In rodents, increases in inducible heat shock protein 70 kDa (HSP-72) stimulated by heat stress limit injury to tissues caused by ischemia/reperfusion [Stojadinovic 1995] or by hemorrhage [Mizushima 2000]. Likewise, inhibition of nitric oxide (NO) production results in significant reduction of local tissue damage, polymorphonuclear neutrophil (PMN) infiltration, and leukotriene B_4 (LTB_4) generation caused by ischemia/reperfusion [Charier 1999]. Mice deficient in inducible NO synthase (iNOS) also demonstrate limited hemorrhage/resuscitation-induced injury [Hierholzer 1998; Moore 1994]. Therefore, remedies that induce HSP-70i and/or inhibit iNOS might prove very useful for reducing hemorrhage/resuscitation-induced injury in man.

The above observations are consistent with the idea that the low oxygen supply resulting from conditions such as ischemia or hemorrhage affects the expression of iNOS, which then influences the expression of other proteins that alter cell viability. It has been shown that hypoxia results in alteration of iNOS, Bcl-2, and P53 mRNA expression in cultured human intestinal epithelial T84 cells and Jurkat T cells, alterations that can be modulated by treatment with a NOS inhibitor [Kiang 2003b]. It is also found that hypoxia increases the activity of caspase-3, an aspartate-specific cysteinyl protease involved in apoptosis, an activity that is blocked by NOS inhibitors [Kiang 2003b].

A full time-course study of the effect of hemorrhage on a series of stress-related proteins such as c-JUN, Kruppel-like factor 6 (KLF6), iNOS, HSP-70i, and hypoxia inducible factor 1α (HIF-1α) has been reported in a hemorrhage mouse model [Kiang 2004b]. Based on Western blot data obtained from mouse lung, jejunum, heart, kidney, liver, and brain, c-JUN, KLF6, iNOS, HSP-70i, and HIF-1α are upregulated. In jejunum, c-JUN protein was overexpressed within 1 h, but levels returned to baseline values 3 h later. KLF6 began to increase significantly 6 h later, reached the maximum at 24 h, and remained at that level at 48 h after hemorrhage. iNOS increased at 6 h, reached a maximum at 12 - 24 h, and returned to baseline values at 48 h. HSP-70i increased at 12 h and remained at elevated levels at 48 h. HIF-1α also increased at 12 h and continued to increase at 48 h. The sequence of protein appearance was c-JUN, KLF6, iNOS, HSP-70i, and HIF-1α. KLF4 (a repressor to iNOS, [Warke, 2003]) and c-FOS (an AP-1 protein) were not detected, and NF-

κB 65 kDa was detected but not affected by hemorrhage. A similar time-course observation on these stress-related proteins was also obtained in lung, kidney, liver, and heart. Sham-treated mouse organs displayed no changes in the basal levels of c-JUN, KLF6, and iNOS. In addition to changes in this series of stress-related proteins listed above, hemorrhage also increases cellular caspase-3 activity [Kiang 2004a] and reduces cellular ATP levels [Chaudry 1973, Chang 2000; Paxian 2003].

2.1 NO and NOS

Like ischemia and reperfusion or hypoxia [Kiang 2003b], hemorrhage increases NO production and NOS overexpression [Kiang 2004b]. The substrate of NO is L-arginine with O_2 and the enzymes involved in this process are either constitutive NOS (cNOS) or iNOS. The chemical biology of NO shows its direct effects and indirect effects [Thomas 2003]. The direct effects are NO reacts with heme-containing proteins, named the primary mode of NO action. These reactions are generally rapid, require low concentrations of NO, and are the genesis of most of the physiological effects of NO. In contrast, the indirect effects, namely the secondary mode of NO action, include formation of N_2O_3, ONOO-, NO_2, and HNO that react with cellular targets and may result in a major configuration change in critical molecules. It has been shown that indirect effects require much higher concentrations of NO than direct effects. It appears that NO produced at low concentrations for short periods primarily mediates direct effects, whereas high local NO concentrations sustained over prolonged periods mediate indirect reactions.

Table 2: Types of NOS and Their Characteristics

NOS	Sizes(kDa)	Chromosome	Features	Tissues
nNOS (NOS1)	160	12	Ca^{2+}-dependent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Gene has no TATA box	Neurons
eNOS (NOS3)	135	7	Ca^{2+}-dependent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Myristonylation and palmitonylation Gene has no TATA box	Endothelium
iNOS (NOS2)	130	17	Ca^{2+}-independent NADPH, FAD, FMN, Heme Calmodulin PKC phosphorylation Gene has a TATA box	Macrophages

NO production is mediated by NOS. NOS can be divided into two major categories: constitutive form (cNOS) and inducible form (iNOS) [Griffith 1995; Nathan 1994].

cNOS includes nNOS and eNOs and it is Ca^{2+}-dependent, whereas iNOS is not. Both cNOS and iNOS contain a reductase domain and an oxidase domain. However, eNOS has myristonylation and palmitonylation sites, whereas nNOS and iNOS do not. The iNOS promoter has a TATA site and differs in that from the cNOS promoters which are TATA-less promoters. The cNOS promoters are GC rich and are regulated primarily by Sp1 and other members of the Sp1-like family. In contrast, NF-κB plays a crucial role in the regulation of iNOS. In the murine iNOS promoter, the downstream NF-κB binding site (-76 to -85 bp) seems the most important one [Xie 1994], however, the upstream NF-κB site (-974 to -960 bp) also seems to have some functionality and cooperativeness with the downstream site [Spink 1995]. For the human iNOS promoter, the NF-κB motif at -5.8 kb is most critical for cytokine-induced promoter activity, whereas the sites at -5.2, -5.5, -6.1, and -8.2 kb have a cooperative effect [Taylor 1998; Marks-Konczalik 1998].

cNOS is thought to generate low levels of NO at submicromolar range for short durations. iNOS generates NO for prolonged periods and at local concentrations as high as 1-5 μM. Since the chemistry and biological outcome depend on the concentration of NO, the proximity of a biological target to the NO source becomes critical. For example, cells or tissues close to macrophages that produce high levels of NO will be subjected to direct and indirect effects due to the primary and the secondary modes of NO actions. In contrast, if they are far from the NO source, they will experience only direct effects as the primary mode of NO action.

2.2 Downregualtion of iNOS

A series of NOS inhibitors have been designed and extensively studied [Salemo 2002]. Among them, L-NNA (N-nitro-L-arginine), L-NMA (N-methyl-L-arginine), L-NIL (N-(1-iminoethyl)-L-lysine, and L-NIO (N-(1-iminoethyl)-L-ornithine) are shown to effectively inhibit iNOS gene expression, NO production, and caspase-3 activity [Moore 1994; Kiang 2003b].

In addition to the use of NOS inhibitors, iNOS deficiency can be caused by deletion of the iNOS gene. This has been shown to reduce or prevent hemorrhage-induced injury and death [Hierholzer 1998]. This result further confirms that iNOS is responsible for the hemorrhage-induced injury. Therefore, downregulation of iNOS by any means will be beneficial to patients suffering from hemorrhage.

Other than iNOS inhibitors and iNOS gene deletion, compounds such as ansamycin can inhibit iNOS activity and gene expression. Both geldanamycin and 17-allylamino-17-demethoxygeldanamycin, members of the ansamycin family, have been demonstrated to reduce NO production, iNOS mRNA, and cytokine-induced activation of the iNOS gene promoter in cultured cells [Murphy 2002], in rats [Pittet 2001], and in mice [Kiang 2004b].

3.0 HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are present in most cells. They represent multigene families that range in molecular size from 10 to 174 kDa. Based on their molecular weights, they are divided into HSP-10, HSP-20, HSP-40, HSP-60, HSP-70, HSP-90, and HSP-110. Table 3 lists the members of each family, their locations, and functions in the cell [Kiang 1998].

Table 3: Members of HSPs and Their Locations and Functions [Kiang 1998]

HSP	Members	Locations	Functions
HSP-110	HSP-110/104	Cytosol/nucleus	Cytoprotection
HSP-90	HSP-90α	Cytosol/nucleus	Endogenous steroid receptor antagonist
	HSP-90β	Cytosol/nucleus	Cytoprotection
	GRP-94	Endoplastic reticulum	Chaperone
HSP-70	GRP-78 (Bip)	Endoplastic reticulum	Chaperone
	HSP-75 (GRP-75)	Mitochondria	Chaperone
	HSP-73	Cytosol/nucleus	Chaperone
	HSP-72	Cytosol/nucleus	Cytoprotection
HSP-60	HSP-60	Mitochondria	Cohort to HSP-75
	HSP-56	Cytosol	Binds to steroid receptors and FK506
HSP-40	HSP-47	Endoplastic reticulum	Collagen chaperone
HSP-20	HSP-27	Cytosol/nucleus	Chaperone; Cytoprotection
HSP-10	HSP-10	Mitochondria	Cohort to HSP-60

3.1 Structure of HSP-72 and Its Functions

The molecular structure of HSP-72 (an inducible form) is very similar to that of HSP-73 (a constitutive form). They both contain a globular unit linked to a β-sheet and an α-helical tube. The globular unit is the ATPase domain (1-386 a.a., 44 kDa); the β-sheet and the α-helix tube (384-543 a.a., 18 kDa) are the peptide binding domain; and the tail (542-646 a.a., 11 kDa) of HSP-73 has the signal peptide of EEVD, but not the tail (542-640 a.a., 10 kDa) of HSP-72. Proteins in the cytoplasm recognize the EEVD of HSP-73 as a chaperone protein, whereas HSP-72 has no such EEVD as a chaperone but instead acts as a cytoprotectant.

3.2 HSP-72 Gene Regulation

HSP-72 gene regulation involves phosphorylation, heat shock transcriptional factor, heat shock elements, and ions.

3.2.1 Heat Shock Elements

Genomic footprinting of the human HSP-70 promoter has revealed that heat stress induces a quick binding of heat shock transcriptional factors (HSFs) to a region encompassing five nGAAn sequences named heat shock elements (HSEs), three of them are GAA (also called perfect HSEs) and other two are GAC and GGG (also called imperfect HSEs). The sequences of HSEs at site 3 and site 4 are dyad symmetrical, and HSFs preferably bind to HSEs at sites 3 and 4 [Kiang 1998]. In mammalian cells, HSEs are usually bound by HSF4 to keep other HSFs away.

3.2.2 Heat Shock Transcriptional Factors

Four different HSFs have been identified in vertebrate: HSF1, HSF2, HSF3, and HSF4 [Morimoto 1996; Nakai 1997]. HSF2 has two isoforms due to splicing: HSF2A and HSF2B [He 2003]. HSFs have a binding

domain, a helical trimerization surface, and a short conserved element. The binding domain is to bind protein and HSEs; the trimerization surface has leucine zipper coiled-coil motifs for trimer formation. There are two genes in mice and humans encoding HSFs. The sizes for human HSF1, HSF2, HSF4, and chicken HSF3 are 529 a.a., 510 a.a., 463 a.a., and 467 a.a., respectively. Human HSF1 can be activated by various stimuli, human HSF2 only by hemin, chicken HSF3 only by heat, while human HSF4 is not activated.

It has been reported that mouse HSF1, chicken HSF3, and mouse HSF4 bind to all 5 HSEs, whereas mouse HSF2 interacts with only 4 HSEs. Activation of HSF1, 2, or 3 upregulates HSP-72 [Kiang 1998]. In contrast, HSF4 is responsible for downregulation of HSP-72 [Nakai 1997].

3.2.3 HSP-72 Autoregulation

Normally, HSFs that reside in the cytosol of mammalian cells are bound to HSPs under unstressed conditions. Under stress conditions such as hemorrhage, HSFs are separated from HSPs. Then, these HSFs are available for phosphorylation by protein kinase C or other serine/threonine kinases. They form homotrimers [Kroeger 1993] or heterotrimers [He 2003]. The trimers enter the nucleus, bind to HSEs located on the promoter region of HSP genes, and become further phosphorylated by HSF kinases. Transcription is then initiated and translation is resulted. Therefore, new HSP-72 is synthesized in the cytosol of cells. Soon, the elevated HSP-72 is bound by cytosolic HSFs. The complex of HSP-72 and HSFs turns off the further increase in HSP-72. The steps of HSFs phosphorylation, trimerization, and its translocation from the cytosol to the nucleus are Ca^{2+}-dependent [Kiang 1994].

3.3 HSP-72 Protein Regulation

HSP-70 family is present in every cell type and tissue under both unstressed and stressed conditions. The inducible form of HSP-70, HSP-72, is induced by physiological stressors, pathological stressors, and environmental stressors. The degree of induction depends on the level and duration of exposure to stressors. The increase usually is transient, but how long it persists is different in various cell types, ranging from hours, days, or weeks. It can be regulated by hormones, intracellular pH, cellular cAMP levels, intracellular concentrations of Ca^{2+}, and activation of protein kinase C, protein kinase A [Kiang 1998; 2003a] and protein tyrosine kinase [Kiang 2004b]. Aging is also known to result in overexpression of HSP-72 that in turn desensitizes Ca^{2+} machinery and turns off new synthesis of HSP-72 [Kiang 1996]. Bioflavonoid such as quercetin prevents HSF1 binding to HSEs, thereby leading to attenuation of HSP-72 gene expression [Murphy 2002].

4.0 INTERACTION BETWEEN HSP-72 AND iNOS

Using immunoprecipitation and immunoblotting analysis, it has been found that HSP-72 forms a complex with iNOS after hemorrhage [Kiang 2004b]. No complex formation is detected between HSP-72 and p53 or Bcl-2 proteins, suggesting that HSP-72 specifically couples to iNOS. Treatment with geldanamycin increases HSP-72 expression and decreases the hemorrhage-induced increase in iNOS expression. The complex formation between HSP-72 and iNOS is still observed. It is possible that the complex formed between HSP-72 and iNOS might decrease the enzymatic activity of iNOS and decrease NO production. As a result of a low level of NO, the injury caused by hemorrhagic shock is markedly diminished.

5.0 RESUSCITATION FLUIDS

Currently, the major cause of death in potentially salvageable battlefield casualties is hemorrhage [Bellamay 1984]. About 20% of these deaths are preventable if the bleeding can be quickly controlled or minimized [Bellamay 1987; Bellamay 1995] and sufficient resuscitation fluid is administered in time to maintain critical tissue perfusion. However, it has been recognized that resuscitation fluids are not innocuous and that they may actually potentiate the cellular injury caused by hemorrhagic shock [Committee on fluid resuscitation for combat casualties 1999]. It has been proposed that some resuscitation fluids may contribute to delayed multiple organ dysfunction [Baue 1998].

The optimal resuscitation fluid is still a matter of debate. In particular questions have been raised about the use of lactated Ringers (LR). LR has been shown to increase neutrophil activation. Hemorrhage elevates mRNA levels of iNOS, KLF6, p-38 MAPK, p53, Bcl-2, and caspase-3 genes in rat lung and ileum. The LR resuscitation does not block their increases. Instead it increases some of them further [Kiang 2004c]. Alam et al. [2002] also reported that 51 genes were altered in rats treated with hemorrhage plus LR resuscitation. The dissection of the role of each of these changes poses a significant challenge.

6.0 ADJUNCT THERAPY AGENTS IN THE RESUSCITATION FLUID

As mentioned above, resuscitation may actually potentiate the cellular injury caused by hemorrhagic shock [Committee on fluid resuscitation for combat casualties 1999] since delayed multiple organ dysfunction and failure occur and mortality results after resuscitation [Baue 1998]. Because iNOS is known to be responsible for the hemorrhage-induced injury and HSP-72 is shown to offer cytoprotection, it is likely that agents or remedies that can reduce iNOS expression and/or increase HSP-72 will be beneficial in conjunction with administration of resuscitation. iNOS inhibitors such as L-NIL, and L-NIO can be potentially useful for this purpose. Likewise, HSP-72 inducers such as herbimycin A, heat stress, ethanol, and ansamycin are also in the list for consideration. To be more precise, ansamycin actually is a HSP-72 inducer as well as an iNOS inhibitor. Therefore, ansamycin and its derivatives should be evaluated for their potential to decrease systemic inflammatory response syndrome, multiple organ dysfunction, and multiple organ failure.

7.0 CONCLUSIONS

Hemorrhage/reperfusion-associated pathophysiology is complicated and poorly understood. Many adverse effects of hemorrhage/resuscitation are common to ischemia/reperfusion and hypoxia/reoxygenation. Resuscitation eventually does not reverse the hemorrhage-induced changes, but instead worsens the changes. The complexity of the cellular response to hemorrhage/resuscitation complicates efforts to design approaches to treat or prevent injury resulting from resuscitation. Nevertheless, an additive in resuscitation fluids, which blocks iNOS, induces HSP-72, and restores ATP depletion, may be potentially therapeutic to salvageable patients.

8.0 PERSPECTIVE

Hemorrhage induces oveexpression of iNOS and HSP-72 proteins, increases in cellular caspase-3 activity [Kiang 2004a], and reduction in ATP [Chaudry 1973; Chang 2000; Paxian 2003]. iNOS overexpression appears early and it leads to the NO production and its direct and indirect effects. HSP-72 overexpression appears 12 h after occurrence of hemorrhage. Because of its late appearance, it is possible that HSP-72 is not serving its usual protective role in hemorrhage and resuscitation, but that it is rather facilitating tissue repair

for salvaging the damage caused by hemorrhage and resuscitation. Treatment with ansamycin is known to increase HSP-72 and reduce iNOS, cellular caspase-3 activity and ATP loss. Taking together the data obtained from ansamycin or agents such as glutamine or crocetin [Van Way 2003], adjunct therapy with novel resuscitation fluid such as ATP-$MgCl_2$ (Nalos 2003) or ethyl pyruvate (Fink 2004) may be a novel approach to address the problems raised from hemorrhagic shock.

Despite the data in the literature, there is little known about the proper time course that these agents should be added to resuscitation fluids after hemorrhage in order to prevent tissue injury. Their pharmacodynamics and pharmacokinetics are still not characterized. More studies are needed to advance the understanding of hemorrhagic shock in order to form a useful therapeutic protocol or a drug formula that can be followed and practiced.

9.0 ACKNOWLEDGMENTS

The work was supported by DOD RAD II STO R. The authors thank colleagues and collaborators participated in this project. The content has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation and/or publication. The views of the authors do not purport to reflect the position of the Department of the Army, Uniformed Services University of The Health Sciences, or the Department of Defense, (para 4-3), AR 360.5.

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