Review

Biology of Hypoxia

Juliann G. Kiang[1, 2, 3], and K.T. Tsen[4]

[1]*Department of Cellular Injury*
Walter Reed Army Institute of Research
Silver Spring, MD 20910
Department of [2]Medicine and of [3]Pharmacology
Uniformed Services University of the Health Sciences
Bethesda, MD 20814
and
[4]*Department of Physics and Astronomy*
Arizona State University
Tempe, AZ 85287, USA

Abstract

Hypoxia is an often seen problem resulting from conditions such as ischemia, hemorrhage, stroke, premature birth, and other cardiovascular difficulties. To find useful remedies that are capable of ameliorating its casualty is an essential effort. Although the underlying mechanisms of the hypoxia-induce injury and cell death are still not fully understood, it has been shown that hypoxia induces nitric oxide (NO) overproduction and inducible nitric oxide synthase (iNOS) overexpression that play important roles in producing injury including increases in polymorphonuclear neutrophils (PMN) infiltration to injured tissues and leukotriene B_4 (LTB_4) generation. Moreover, it has been evident that transcription factors responsible for iNOS expression are also altered by hypoxia. Hypoxia also increases intracellular Ca^{2+} concentration, tumor necrosis factor-α, lipid peroxidation, prostaglandin E_2 production, activity of caspase-3 and -9, and release of cytochrome c from mitochondria, apoptosis inducible factor, and endonuclease G. However, it has been shown that downregulation of iNOS can limit cell injury caused by hypoxia. In our laboratory, we have found that treatment with either iNOS inhibitors or iNOS siRNA inhibits iNOS expression, reduces lipid peroxidation, apoptosome formation, and cellular caspase-3 activity, preserves cellular ATP levels, and increases cell survival. Therefore, iNOS inhibition may be a novel mechanism for protection from hypoxia-induced injury and cell death.

Key Words: hypoxia, iNOS, caspase, ATP, calcium, NO, free radical

Introduction

Hypoxia results from conditions such as ischemia, hemorrhage, stroke, premature birth, and other cardiovascular difficulties, among which hemorrhagic shock is the leading cause of death and complications in combat casualties and civilian settings. It has been shown to cause systemic inflammation response syndrome (SIRS), multiple organ dysfunction (MOD), and multiple organ failure [(MOF), see ref. 7]. It is an often seen problem and to find useful remedies that are capable of ameliorating its casualty is an essential effort in combat medicine, trauma injury, and cardiovascular-related scenarios. Hypoxia has been shown to lead to increases in intracellular free calcium concentration ($[Ca^{2+}]_i$), 5-lipoxygenase, lipid peroxidation, cycloxygenase (COX), constitutive nitric oxide synthase (cNOS), leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), interlukins, tumor necrosis factor-α (TNF-α), caspases, complement activation, kruppel-like factor 6 (KLF6), inducible nitric oxide synthase (iNOS), heat shock protein 70 kDa (HSP-70), and hypoxia-inducible factor-1α [(HIF-1α), see refs. 15, 28, 37, 58, 60, 73, 81, 86]. The sequence of their occurrence provides the useful information for studying the mechanisms underlying the hypoxia-induced injury as well as

Corresponding author: Dr. Juliann G. Kiang, Department of Cellular Injury, Walter Reed Army Institute of Research, Silver Spring, MD 20910-7500, U.S.A. E-mail: Juliann.Kiang@na.amedd.army.mil (or) kiangj@comcast.net
Received: August 7, 2006; Accepted: August 29, 2006.

therapeutic targets to prevent or ameliorate the injury. In this paper, the calcium effect and the role of iNOS, Bcl-2, p53, caspases, ATP, and free radicals in hypoxia-induced injury are discussed in details.

Calcium Effect

Intracellular free Ca^{2+} has been long recognized as a ubiquitous second messenger in variety of physiological systems. The external signal is transmitted into the cells through intracellular free Ca^{2+}. Increases in resting $[Ca^{2+}]_i$ trigger many cell functions, including metabolism, growth, differentiation, hormonal secretion, gene expression, protein synthesis, and cell movement (44, 45, 57, 94).

The resting $[Ca^{2+}]_i$ is regulated at three different levels: 1) at cell membrane; 2) at the intracellular Ca^{2+} pools in the cytoplasm; and 3) by Ca^{2+}-binding proteins in the cytoplasm and the nucleus.

First, there are Na^+/Ca^{2+} exchanger, Ca^{2+}/H^+ antiporter, Ca^{2+}-ATPase pumps, and Ca^{2+} channels (voltage-gated, messenger-operated, and receptor-operated) present at the cell membrane. Normally, activation of the Na^+/Ca^{2+} exchanger stimulates the import of three molecules of Na^+ and the export of two molecules of Ca^{2+}. However, this exchanger is concentration-driven, so the Na^+ and Ca^{2+} concentration gradients between the cytoplasm and the extracellular space can reverse the direction of Na^+/Ca^{2+} exchange. The Ca^{2+}/H^+ antiporter takes in two molecules of H^+ and expels one molecule of Ca^{2+}. This antiporter is pH-sensitive (34). Ca^{2+}-ATPase pumps are energy-driven and remove Ca^{2+} from the cell. Voltage-gated Ca^{2+} channels are activated by changes in membrane potential. There are L-type, N-type, and T-type. Messenger-operated Ca^{2+} channels are activated by inositol 1,4,5-trisphosphate (IP3) or cAMP-stimulated protein kinase (PKA); receptor-operated Ca^{2+} channels are activated by ligand-binding to receptors on the membrane.

Second, there are intracellular Ca^{2+} pools in the cytoplasm. The increase in $[Ca^{2+}]_i$ induces Ca^{2+} mobilization from intracellular Ca^{2+} pools to further elevate $[Ca^{2+}]_i$ (i.e., Ca^{2+}-induced Ca^{2+} increase). However, Ca^{2+} sequestration into the intracellular Ca^{2+} pools to lower $[Ca^{2+}]_i$ also takes place.

Third, Ca^{2+}-binding proteins are present in the cytoplasm and nucleus. The glutamic acid- and phenylalanine-rich proteins, like calmodulin and S100 proteins, are considered to exert Ca^{2+}-dependent actions in the nucleus or the cytoplasm. The Ca^{2+}/phospholipid binding proteins are classified into two groups, the annexins and the C2 region proteins. These proteins, distributed mainly in the cytoplasm, translocate to the plasma membrane in response to an increase in cytosolic Ca^{2+} and function in the vicinity of the membrane (65).

Hypoxia has been shown to perturb calcium homeostasis. Cyanide-induced chemical hypoxia causes an increase in $[Ca^{2+}]_i$ in PC12 cells (32, 54), rabbit carotid body chemoreceptors (8, 79), rat ventricular myocytes (20), rat osteoclasts (90), Leishmania donovani promastigotes (72), and human epidermoid A-431 cells (43). Hypoxia caused by exposure of rat thyroid FRTL-5 cells to 5% O_2 also induces a significant increase (46). In PC12 cells, the increase is believed to be activated by voltage-gated Ca^{2+} channels (32). In rabbit carotid body chemoreceptors, it is due to inhibition of Na^+/Ca^{2+} exchanger (8, 79). In Leishmania donovani promastigotes, it results from a Ca^{2+} mobilization from intracellular Ca^{2+} pools (72). In human epidermoid A-431 cells, the $[Ca^{2+}]_i$ increase is because of an increased Na^+ influx that activates the reversed mode of the Na^+/Ca^{2+} exchanger, thereby leading to an increased Ca^{2+} entry. The Ca^{2+} influx induces, in turn, a Ca^{2+}-stimulated mobilization of Ca^{2+} from intracellular Ca^{2+} pools through ryanodine receptor channels that are sensitive to ionomycin but not to bradykinin or monensin (43). It should bear in mind that hypoxia decreases IP3 (43), which is distinctly different from that observed in heat stress (43, 44).

It is believed that an abrupt increase in $[Ca^{2+}]_i$ is associated with cell death (21, 44, 68, 92) mediated by activation of Ca^{2+}-dependent proteases (24). However, Snyder *et al.* (85) reported that the hypoxia killing of hepatocytes correlates closely with changes in the mitochondrial membrane potential. In contrast, Sakaida *et al.* (76) concludes that hypoxia kills cultured hepatocytes because of an alteration in the interaction between the cytoskeleton and the plasma membrane, i.e. membrane blebbing. Regardless the controversy, the increase in $[Ca^{2+}]_i$ is known to turn on many Ca^{2+}-dependent enzymes that trigger the down-stream cascade reactions, thereby probably leading to either the necrosis or the apoptosis (43, 44). Remedies such as overexpression of HSP-70i are shown to inhibit the hypoxia-induced increase in $[Ca^{2+}]_i$ and to increase the cell survival (39).

NO and NOS

NO is generated by L-arginine reacting with O_2 in the process involving with catalyzing activity of cNOS or iNOS. The chemical biology of NO includes its direct effects and indirect effects (81, 91). The direct effects, also named the primary mode of NO action, are NO reacts with heme-containing proteins. These reactions are generally rapid, require low concentrations of NO, and are the genesis of most of the physiological effects of NO such as vasodilation. On the contrary, the indirect effects, namely the secondary mode of NO action, include formation of

Table 1. Transcriptional factors that binds to the promoter of iNOS gene.

Motif for Transcriptional factors	Function
4 CCACC motifs for KLF6	Stimulates iNOS gene
Unidentified motif for KLF4	Inhibits iNOS gene
2 AP-1 binding sites for AP-1 proteins	Stimulates iNOS gene
10 NF-κB binding sites for NF-κB	Stimulates iNOS gene

KLF6: Kruppel-like factor 6; KLF4: Kruppel-like factor 4; AP: activation protein; NF-κB: nuclear factor kappa B; iNOS: inducible nitric oxide synthase

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 at a micro molar level sustaining over prolonged periods mediate indirect reactions and cause pathophysiological effects such as organ dysfunction and failure. NO reacts with NO_2 to form N_2O_3 resulting in nitrosative stress, while it does with O_2^- to form $OONO^-$ and NO_3^- causing oxidative stress. NO is also known to react with biologic metals, to modulate catalase and GSH, and to alter cell sensitivity to cell death (91).

It is evident that hypoxia upregulates NOS expression. As a result, NO production is increased. Hierholzer and Billiar (27) reported that in hemorrhage/resuscitation-induced injury model, the induction of inducible nitric oxide (iNOS), along with cyclooxygenase (COX)-2, and CD14 are up-regulated. The early immediate response genes iNOS and COX-2 promote the inflammatory response by the rapid and excessive production of NO and prostaglandins. In our laboratory, we have found that hypoxic human intestinal epithelial T84 cells and hypoxic human Jurkat T cells display increases in mRNA of iNOS and HSP-70, while an increase in p53 mRNA is shown in only T84 cells and an increase in Bcl-2 mRNA in only Jurkat T cells (47).

In *in vivo* studies, it is evident that inhibition of NO production by N^{ω}-nitro-L-arginine (LNNA), an irreversible cNOS inhibitor and a reversible iNOS inhibitor (40, 77), results in significant reduction of local tissue damage, PMN infiltration and LTB_4 generation caused by ischemia/reperfusion (89). Similar observation is found with heat stressed rats after ischemia and perfusion (86). The hemorrhage/resuscitation-induced injury is also reported to be prevented in iNOS knockout mice or by treatment with L-N^6-(1-iminoethyl)-lysine (L-NIL), a selective inhibitor of iNOS (28). Treatment of mice (37) and rats (73) with geldanamycin effectively inhibits iNOS while increases HSP-70i. The inhibitory action of geldanamycin is contributed by its capability to suppress the transcriptional factors of iNOS gene, including c-jun, KLF4, KLF6, and NF-κB (Table 1). Other treatments with such 17β-estradiol (102), androstenediol (42, 82), pyruvate (17), or resuscitation with lactated Ringer solution (41) have been shown to reduce iNOS caused by hemorrhage and resuscitation. In contrast, Gabbai *et al*. (23) reported that inhibition of iNOs by L-NIL intensifies injury and functional deterioration in autoimmune interstitial nephritis. Nevertheless, during hypoxia in neuronal nuclei from newborn piglets LNNA pretreatment prevents the hypoxia-induced increase in Bcl-2 protein and DNA fragmentation (106).

In *in vitro* studies, hypoxia significantly increases iNOS in human intestinal epithelial T84 cells and human Jurkat T cells. LNNA treatment effectively inhibits hypoxia-induced increase in iNOS (47).

Bcl-2 and p53

In addition, hypoxia also alters expression of Bcl-2 and p53. Expression of Bcl-2 is usually accompanied by inactivation of tumor suppressor protein p53, which frequently leads to tumor progression (25, 103). Bcl-2 is homologous to CED-9 observed in *C. elegans* (97). In contrast to stimulatory cell growth and blockade of cell death of Bcl-2, it has been reported that p53 inhibits cell growth and promotes differentiation and programmed cell death and is controlled by transcriptional, translational, and post-translational regulation (105). Hypoxia resulted from ischemia has shown to activate p53 in neurons (105). In our laboratory, we found that hypoxia induced by NaCN treatment increases p53 mRNA in T84 cells and Bcl-2 mRNA in Jurkat T cells. In both cells, treatment with LNNA blocked Bcl-2, but increased p53, a result perhaps very favorable to stop tumor progression (47). In rat liver, hypoxia caused by trauma-hemorrhage-resuscitation decreases both Bcl-2 and p53 (42) whereas in rat small intestine and lung, hypoxia increases both of them (41). These results suggest that Bcl-2 and p53 responding to hypoxia are organ- and cell-specific.

Table 2. Apoptosis pathways (29, 31).

Pathways	Components
Extrinsic pathway	Fas/APO-1/CD95 with FasL
	TNFR with TNF-α
	FADD
	Caspase-8
	Bid
Intrinsic pathways	Cytochrome c
	Apaf-1
	Apoptosome
	Caspase-9, -3, -7, -2, -6, -8,-10
AIF	DNA fragmentation
EndoG	DNA fragmentation

Apoptosome is a complex that consists of cytochrome c, Apaf-1, and caspase-9. FasL: Fas ligand; TNFR: Tumor necrosis factor receptor; FADD: Fas-associated death domain; AIF: Apoptosis inducible factor; EndoG: Endonuclease G

Table 3. Caspases (29).

Name	Procaspase domain	Preferred substance sequence	Function
Caspase-1	CARD-(119 ~ 297)-(317 ~ 404)	WEHD or YEHD	Inflammation
Caspase-2	CARD-(152 ~ 361)-(331 ~ 435)	DEHD or VDVAD	Apoptosis
Caspase-3	-(28 ~ 175)(175 ~ 277)	DEVD or DNQD	Apoptosis
Caspase-4	CARD ~ 270-(290 ~ 377)	LEVD or (W/L)EHD	Inflammation
Caspase-5	CARD(1 ~ 311)-(331 ~ 418)	(W/L)EHD	Inflammation
Caspase-6	-(23 ~ 179)-(194 ~ 293)	VEID or VEHD	Apoptosis
Caspase-7	-(23 ~ 198)-(198 ~ 303)	DEVD	Apoptosis
Caspase-8	DED-DED-(216 ~ 374)-(385 ~ 479)	(I/L)ETD	Apoptosis
Caspase-9	CARD(1 ~ 315)-(331 ~ 416)	LEHD	Apoptosis
Caspase-10	DED-DED-(219 ~ 415)(415 ~ 521)	IEAD	Apoptosis
Caspase-14	-(? ~ ?)-(? ~ 242)	?	Keratinocyte Differerntiation?

Numbers in parenthesis are the positions of amino acids. Caspase-14 is not fully characterized. CARD: Caspase-recruitment domain; DED: Death effector domain

Caspases

Caspases are the executioners of apoptosis. Apoptosis is characterized by a series of morphological changes, including chromatin condensation, membrane blebbing and cell shrinkage. As a result, cells are fragmented and become vasicular bodies that are taken up by macrophages (2). Caspases are cysteine aspartic acid-specific proteases that cleave amino acid sequence-motif located N-terminal to a specific aspartic acid residue. Caspases can be broadly divided into 2 functional subgroups (Table 2): [1] those that are activated during apoptosis (caspases -2, -3, -6, -7, -8, -9, -10); and [2] those that have been implicated in the processing of proinflammatory cytokines during immune responses (caspases -1, -4, -5). Caspases are synthesized as inactive zymogens (i.e. procaspases) that require proteolytic cleavage to form the large and small subunits of the active enzyme, suggesting the caspases either become activated through auto-proteolysis or by other activated caspases (29).

Table 3 lists that apoptosis is mediated by [1] the extrinsic pathway, [2] the intrinsic pathway, [3] the apoptosis inducible factor-induced pathway, and/or [4] the EndoG-involved pathway. The extrinsic pathways starts with either Fas ligand binding onto Fas/APO/CD95 or TNF-α binding onto TNF receptors on the cell membrane. The intrinsic pathway is initiated by cytochrome c released from mitochondria to the cytoplasm. Apaf-1 (100) in cytoplasm contains an N-terminal caspase-recruiment domain (CARD), a central nucleotide-binding domain (homolog of CED-4 in *C. elegans*), and twelve to thirteen WD-40 repeats at the C terminus of the molecule. The CARD of

Apaf-1 is replaced with cytochrome c (31). Then this modified Apaf-1 recruits and activates procaspase-9 through the interactions of CARD found on both proteins. The complex, namely apoptosome, activates caspase-3 and -7. Subsequently, caspase-3 activates caspase-2, -6, -8, and -10 (29). AIF and EndoG are two potential executors of caspase-independent cell death when they translocate from mitochondria to the nucleus (6), although controversy is present (4, 5, 10, 16, 18, 87, 88). In *C. elegans*, AIF is a component of the caspase-dependent mitochondrial pathway, which also involves the mitochondrial EndoG (98). However, recent data from EndoG deficient mice do not support a relevant role of this protein in mammalian cell death and DNA processing (30).

It has been shown that caspase-3 activity is increased by directly lowering oxygen tension (19, 47), ischemia (12, 67, 99, 101), hemorrhage (11, 38, 49, 56, 59, 61, 104), and in other pathological conditions (1, 61, 63, 71, 83, 98). In murine hemorrhage model (38), the hemorrhage-induced hypoxia increases caspase-3 within 1 h after hemorrhage in jejunum, lung, heart, and kidney, reaches the maximal levels between 3-6 h, and returned to the basal level in jejunum and heart, but not lung and kidney. The level of the lung caspase-3 increases continuously; the kidney caspase-3 returns to the basal level at 24 h and yet increases to the maximal level at 48 h. Unlike jejunum, lung, heart, and kidney, the brain tissue shows a delayed increase in caspase-3 at 24 h and remains there at 48 h.

Interventions that lead to inhibition of caspase-3 activity (104) such as treadmill exercise (49), hypertonic saline resuscitation (61), or LR resuscitation (41) have been shown to reduce tissue apoptosis and brain damage in hemorrhaged animals. It is also evident that treatment of cells (47) or animals (37) with iNOS inhibitors results in decreased cellular caspase-3 activity. We found that treatment of rodents with either androstenediol (42) or geldanamycin (37, 38) limits the expression of iNOS and caspase-3. Furthermore, when human small intestinal cells are transfected with iNOS gene, the results from experiments with immunoblotting and immunohistostaining indicate that iNOS protein, caspase-3, caspase-9, and cytosolic cytochrome c are increased. When these cells are transfected with iNOS siRNA, no increases in iNOS, caspase-3, caspase-9, and cytosolic cytochrome c are observed. These results imply that the cellular level of iNOS is positively correlated with caspase-3 enzymatic activity. This conclusion is corroborated by previous findings (47) in human intestinal epithelial and T cells which demonstrate that pharmaceutical manipulation of iNOS results in changes in caspase-3 expression. Figure 1 represents a diagram depicting the hypoxia-induced molecular abnormalities. Hypoxia increases KLF6 and NF-κB and decreases KLF4, resulting in an increased expression of iNOS. This increase in iNOS leads to increase NO production that reacts with O_2^- to form $ONOO^-$. Then, peroxynitrite makes mitochondria swell up and release cytochrome c that subsequently is caged by Apaf-1 and caspase-9 to form apoptosome. The apoptosome, therefore, activates caspase-3, leading to apoptosis.

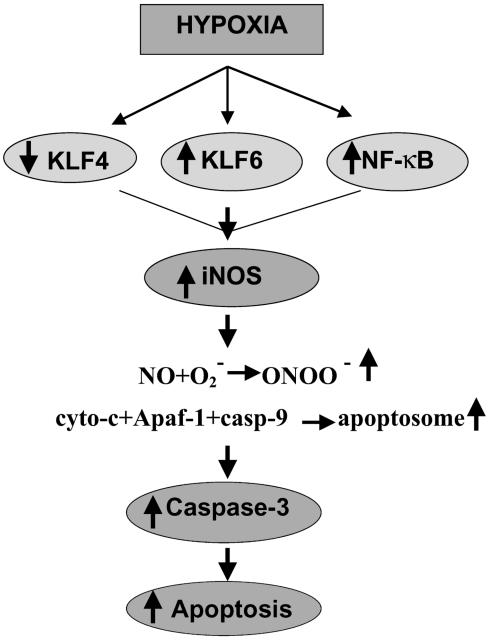

Fig. 1. Schematic representation depicting the hypoxia-induced molecular abnormalities. Hypoxia increases KLF6 and NF-κB and decreases KLF4, resulting in an increased expression of iNOS. This increase in iNOS leads to increase NO production that reacts with O_2^- to form $ONOO^-$. Then peroxynitrite makes mitochondria swell up and release cytochrome C that subsequently is caged by Apaf-1 and caspase-9 to form apoptosome. The apoptosome, therefore, activates caspase-3, thereby leading to apoptosis. KLF4: Kruppel-like factor 4; KLF6: Kruppel-like factor 6; NF-κB: nuclear factor-kappa B; iNOS: inducible nitric oxide; cyto-c: cytochrome c; casp-9: caspase-9; ↑: increase; ↓: decrease

It has been reported that the apoptosis-induced membrane blebbing results from caspase-3-mediated activation of Rho-associated kinase 1 (ROCK 1). ROCK 1 is cleaved by caspase-3 at a conserved DETD1113/G sequence and its carboxy-terminal inhibitory domain is removed. The inactive ROCK 1 becomes active and phosphorylates myosin light chain (MLC). As a result, cell contraction and membrane blebbing occur in apoptotic cells (13, 66, 80).

Bahi *et al*. (6) report that hypoxia induces DNA fragmentation in rat differentiated cardiomyocytes. This DNA fragmentation is contributed by EndoG and AIF but not caspases. It should also bear in mind

that apoptosis also can be initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome (55).

ATP

ATP has been recognized as a universal currency of free energy in biological system since 1941. The energy is stored in the bond between O and P in which the free energy is 7.3 kcal/mol. It is known that Ca^{2+} and Na^{+} break the high-energy bond of the ATP molecule, whereas K^{+} and Mg^{2+} help rebuild it (36, 74). Other laboratories (48, 69, 70, 96) and our laboratory (36) report that hypoxia caused by hemorrhage induces cellular ATP depletion. We have found that hemorrhage decreased ATP levels in mouse jejunum, lung, heart, kidney, and brain tissue lysates. ATP levels in brain and kidney tissues return to basal levels, whereas in jejunum, lung and heart, the ATP levels remain below the basal levels (36). Although the hemorrhage-associated low levels of ATP in jejunum, lung, and heart have been proposed to be responsible for the delayed hemorrhage manifestations such as MOD and MOF, the exact mechanism(s) are not known.

The decrease in cellular ATP levels after hemorrhage is caused at least in part by decreases in the enzymatic activity and the protein expression of pyruvate dehydrogenase (PDH), an enzyme that catalyzes the oxidative decarboxylation of pyruvate to generate acetyl-coenzyme A, thereby linking glycolysis to the TCA cycle with respect to substrate. These hemorrhage-induced decreases are not likely related to the increase in caspase-3 because iNOS gene transfected cells exhibit the increased caspase-3 activity and protein level without altering the levels of PDH and ATP (36). However, the possibility that other caspases are involved in PDH metabolism can not be ruled out. Furthermore, whether enzymes other than PDH are altered by hemorrhage remains to be elucidated.

The documented loss of ATP caused by hypoxia is believed to play a central role in tissue pathology. Figure 2 presents a model for the interaction between hypoxia-induced changes. On the basis of our data and those from other laboratories (96), hemorrhage decreases the PDH enzymatic activity and its protein levels, which results in decreased intracellular ATP. Low intracellular ATP levels cause mitochondria disruption. Because apoptosis requires energy, if the levels of cellular ATP remain at levels higher than 15% of baseline, then apoptosis ensues. If the cellular ATP levels fall below 15% of baseline, then necrosis ensues (14, 50-52).

Interference with the synthesis of ATP in mitochondria results in cell apoptosis and therefore, inhibition of ATP loss has been shown to prevent hemorrhage-associated clinical manifestations (7, 35).

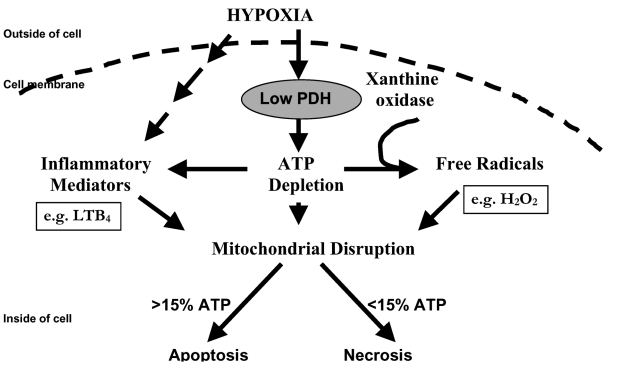

Fig. 2. Schematic representation of model for ATP after exposure to hypoxia. Hypoxia increase inflammation mediators such as LTB_4 and decreases pyruvate dehydrogenase (PDH) activity, which leads to low ATP levels. Low ATP levels and increased inflammation mediators cause mitochondrial disruption that results in either apoptosis or necrosis depending on the level of ATP left in the cell. Concurrently, Low ATP levels enhanced free radical formation that contributes to the mitochondrial disruption.

Addition of ATP-$MgCl_2$ (64) or ethyl pyruvate (17, 22) into resuscitation fluids or treatment with geldanamycin (36) have been shown to maintain tissue ATP levels and limit tissue injury. Treatment with geldanamycin prevents the hemorrhage-induced ATP loss and organ injury probably by inducing the increased expression of HSP-70i and PDH and activating PDH (36).

Free Radicals

Free radicals are molecules that contain unpaired electrons. The unpaired electron is highly reactive. It can either causes oxidative damage to the molecules or is passed from one molecule to another turning the recipient into a free radical (26). Figure 2 depicts that the hypoxia-induced cellular ATP depletion promotes formation of free radicals produced from xanthine with help of the catalyzing activity of xanthine oxidase. It has been shown to have H_2O_2, O_2^{-}, and OH· produced (3). They tend to react with NO to form N_2O_3, $ONOO^{-}$, NO_2, and HNO that react with cellular targets and may result in a major configuration change in critical molecules (91). Depending on the duration of exposure to the assaults and the type of assaults, the presence of NO can enhance or retard toxicity of the reactive oxygen species [(ROS), see ref. 84, 91]. These ROS and reactive nitrogen species (RNS) are thought to cause mitochondrial disruption so that cytochrome c, AIF, or EndoG translocate to the cytoplasm. Then, cytosolic cytochorme c binds to Apaf-1 and caspase-9 to form apoptosome that activates other caspases to initiate apoptosis (29, 31).

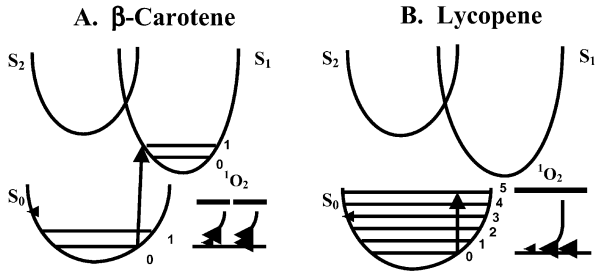

Fig. 3. A proposed energy transfer process suggested by our experimental results in the neutralization of singlet oxygen molecules by β-carotene (A) and lycopene (B). In the process, two singlet oxygen molecules simultaneously lose their excitation energy to β-carotene which is excited to the first excited vibronic state S_1, whereas one singlet oxygen molecule loses its excitation energy to lycopene which is excited to the ground excited vibronic state S_0. The results explain at least in part why lycopene is more effective than β-carotene in neutralizing singlet oxygen.

On the other hand, AIF and EndoG induce DNA fragmentation and micronuclei formation, resulting in occurrence of apoptosis (6, 29, 31). In addition, we have found that hypoxia increases lipid peroxidation in human intestinal epithelial T84 cells and human Jurkat T cells (Kiang, unpublished data).

Removal or neutralization of the free radicals can, therefore, protect against injury induced by harmful assaults. Other benefits are, for instance, that free radicals might underlie the aging process itself because low caloric intake that reduces the generation of free radicals has been shown to increase the life span in *C. elegans* (53) and others (33). Antioxidants such as vitamine C, vitamine E, chlorpromazine, mepacrine, dibucaine, butylated hydroxytoluene, butylated hydroxyanisole, propylgallate, zinc, and allopurinol have all been claimed to have the ability to inhibit free radical-mediated processes. Recently, β-carotene, and lycopene are shown to be good antioxidants. It is reported that lycopene is 20 times more effective than β-carotene in this regard. We have been studied both of them on neutralizing the free radicals.

Energy transfer processes between β-carotene, lycopene and singlet oxygen ($^1O_2$) are studied by ultrafast Raman spectroscopy. Figure 3 shows that during the neutralization of singlet oxygen by β-carotene the excitation energy of singlet oxygen is transferred directly to the first excited electronic state S_1 of β-carotene. In contrast, the excitation energy of singlet oxygen is transferred directly to the ground excited vibronic state S_0 of lycopene (93). Our experimental data provide [1] the first direct experimental elucidation of energy transfer processes in such important biological systems and [2] an explanation at least in part why lycopene is a more potent antioxidant than β-carotene in the neutralization of singlet oxygen.

Conclusion

Hypoxia-associated pathophysiology is complicated and elusive yet conspicuously significant. Many adverse effects of hypoxia are commonly observed under conditions generated by ischemia and hemorrhage. Like reperfusion and resuscitation, reoxygenation eventually does not completely reverse the hypoxia-induced changes. The complexity of the cellular response to hypoxia complicates efforts to design approaches to treat or prevent injury resulting from reoxygenation. Nevertheless, an additive in the reoxygenation process, which can induce HSP-70i, block iNOS, restore ATP depletion, and reduce caspases, may be potentially therapeutic to salvageable patients suffering from hypoxia.

Hypoxia induces overexpression of iNOS and HSP-70i proteins, increases in cellular caspase-3 activity (38), and reduction in ATP (36, 48, 69, 70, 96). iNOS over-expression appears relatively early and it leads to the NO production and its direct and indirect effects. HSP-70i over-expression appears 12 h after occurrence of hypoxia (37). Because of its late appearance, it is possible that this HSP-70i is not serving its usual protective role in hypoxia, but that it is rather facilitating tissue repair for salvaging the damage caused by hypoxia. The continuous increase in caspase-3 in lung, a relapse in kidney and a delayed elevation of brain caspase-3 with the continuous ATP depletion in small intestine, lung and heart 24 h after hemorrhage-induced hypoxia may explain the occurrence of MOD and MOF. However, a pre-treatment to induce HSP-70i overexpression and/or inhibit iNOS induction prior to hypoxia have been proven to prophylactically prevent the resulting injury (35, 42). Other pathways such as PI3-K activation also have been suggested to reduce the hypoxia-induced cell death (62). On the other hand, its post-treatment is still useful in order to minimize the morbid sequelae such as MOD and MOF. Most importantly, it is known that the hypoxia-induced iNOS overexpression causes caspases-activated cell death. iNOS inhibitors and siRNA may be of value in reducing pathology resulting from hypoxia-associated disorder and imbalance.

Perspective

Hypoxia resulted from ischemia, hemorrhage, or other cardiovascular problems is a common scenario in the clinical practice of medicine and occurs in virtually all organ systems. Its hallmarks are relatively consistent across patient populations and organ systems. In our murine model (36-38), treatment with

geldanamycin is known to increase HSP-70i and PDH and reduce iNOS, cellular caspase-3 activity and ATP loss. Though the underlying mechanism is not completely unfolded, geldanamycin treatment inhibits iNOS by inhibiting KLF6, induces HSP-70i by activating HSF1, and elevates PDH protein and its enzymatic activity. HSP-70i is shown to complex with iNOS and its transcription factor KLF6 and the key enzyme of ATP synthesis PDH. In human cultured cells, similar results obtained from experiments with HSP-70 gene transfer or iNOS siRNA treatment are observed. Understanding the interaction between these proteins may provide an insight for a therapeutic design to prevent the hypoxia-induced injury and cell death. Taking together the data obtained from geldanamycin, HSP-70 gene transfer, iNOS siRNA, or other agents such as ethanol (9, 75, 78, 95), glutamine or crocetin (96), adjunct therapy with the optimal resuscitation fluid containing ATP-$MgCl_2$ (64) or ethyl pyruvate (17, 22) may be a novel approach to address the problems raised from assaults under circumstances of hypoxia or hypoxia/reoxygenation.

Acknowledgments

The work was supported by DOD RAD II STO C. The authors thank Drs. Phillip D. Bowman, Baiteng Zhao, James L. Atkins, George C. Tsokos, Xinyue Lu, Lindita S. Takabu, Timothy B. Bentley, Yansong Li, Xuan Z. Ding, Vishal G. Warke, Sherry D. Fleming, Alex Stojadinovic, Terez Shea-Donohue, and Robert C. Smallridge for their outstanding effort in the projects of NaCN, ischemia/reperfusion, and hemorrhage/ resuscitation.

Animal Ethics

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, NRC Publication, 1996 edition.

Disclaimer

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the US Department of the Army, the Uniformed Services University of The Health Sciences, or US Department of Defense, (para 4-3), AR 360.5.

References

1. Adamus, G. Autoantibody-induced apoptosis as a possible mechanism of autoimmune retinopathy. *Autoimmun Rev*. 2: 63-68, 2003.
2. Adrain, C. and Martin, S.J. The mitochondrial apoptosome: A killer unleashed by the cytochrome seas. *Trends Biochem. Sci*. 26: 390-397, 2001.
3. Aitken, R.J., Buckingham, D. and Harkiss, D. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *J. Reprod. Fertil*. 97: 441-450, 1993.
4. Arnoult, D., Gaume, B., Karbowski, M., Sharpe, J.C., Cecconi, F. and Youle, R.J. Mitochondrial release of AIF and EndoG requires caspase activation downstream of Bax/Bak-mediated permeabilization. *EMBO J*. 22: 4385-4399, 2003.
5. Arnoult, D., Parone, P., Martinou, J.C., Antonsson, B., Estaquier, J. and Ameisen, J.C. Mitochondrial release of apoptosis-inducing factor occurs downstream of cytochrome c release in response to several proapoptotic stimuli. *J. Cell Biol*. 159: 923-929, 2002.
6. Bahi, N., Zhang, J., Llovera, M., Ballester, M., Comella, J.X. and Sanchis, D. Switch from caspse-dependent to -independent death during heart development: Essential role of EndoG in ischemia-induced DAN processing of differentiated cardiomyocytes. *J. Biol. Chem*. Doi/10.1074/jbc.M601025200, 2006.
7. Baue, A.E., Durham, R. and Faist, E. Systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), multiple organ failure (MOF): are we winning the battle? *Shock* 10: 79-89, 1998.
8. Biscoe, T.J. and Duchen, M.R. Measurements of intracellular Ca^{2+} in dissociation type I cells of the rabbit carotid body. *J. Physiol. (London)* 416: 421-434, 1989.
9. Calabrese, V., Testa, G., Ravagna, A., Bates, T.E. and Stella, A.M. HSP70 induction in the brain following ethanol administration in the rat: regulation by glutathione redox state. *Biochem. Biophys. Res. Commun*. 269: 397-400, 2000.
10. Cande, C., Vahsen, N., Garrido, C. and Kroemer, G. Apoptosis-inducing factor (AIF): caspase-independent after all. *Cell Death Diff*. 11: 591-595, 2004.
11. Cho, N.H., Lee, J.D., Cheong, B.S., Choi, D.Y., Chang, H.K., Lee, T.H., Shin, M.C., Shin, M.S., Lee, J. and Kim, C.J. Acupuncture suppresses intrastriatal hemorrhage-induced apoptotic neuronal cell death in rats. *Neurosci. Lett*. 362: 141-145, 2004.
12. Cho, S., Liu, D., Fairman, D., Li, P., Jenkins, L., McGonigle, P. and Wood, A. Spatiotemporal evidence of apoptosis-mediated ischemic injury in organotypic hippocampal slice cultures. *Neurochem. Int*. 45: 117-127, 2004.
13. Coleman, M.L., Sahai, E.A., Yeo, M., Bosch, M., Dewar, A. and Olsonm, M.F. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK 1. *Nature Cell Biol*. 3: 339-345, 2001.
14. Comelli, M., Di Pancrazio, F. and Mavelli, I. Apoptosis is induced by decline of mitochondrial ATP synthesis in erythroleukemia cells. *Free Radic. Biol. Med*. 34: 1190-1199, 2003.
15. Conde, A.G., Lau, S.S., Dillmann, W.H. and Mestril, R. Induction of heat shock proteins by tyrosine kinase inhibitors in rat cardiomyocytes and myogenic cells confers protection against simulated ischemia. *J. Mol. Cell Cardiol*. 29: 1927-1938, 1997.
16. Cregan, S.P., Fortin, A., MacLaurin, J.G., Callaghan,S.M.,Cecconi, F., Yu, S.W., Dawson, T.M., Dawson, V.L., Park, D.S., Kroemer, G. and Slack, R.S. Apoptosis-inducing factor is involved in the regulation of caspase-independent neuronal cell death. *J. Cell Biol*. 158: 507-517, 2002.
17. Das, U.N. Pyruvate is an endogenous anti-inflammatory and anti-oxidant molecule. *Med. Sci. Monit*. 12:RA79-84, 2006.
18. Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prevost, M.C., Leber, b., Andrews, D., Penninger, J. and Kroemer, G. Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *FASEB J*. 14: 729-739, 2000.
19. Eigel, B.N., Gursahani, H. and Hadley, R.W. The Sodium-Calcium Exchanger Plays a Key Role in Inducing Apoptosis Following

Prolonged Hypoxia in Cultured Guinea Pig Ventricular Myocytes. *Am. J. Physiol. (Heart Circ. Physiol.)* 287: H1466-H1475, 2004.
20. Eisner, D.A., Nichols, C.G., O'Neill, S.C., Smith, G.L. and Valdeolmillos, M. The effects of metabolic inhibition on intracellular calcium and pH in isolated rat ventricular cells. *J. Physiol. (London)* 411: 393-418, 1989.
21. Farber, J.L. The role of calcium in cell death. *Life Sci*. 29: 1289-1295, 1981.
22. Fink, M.P. Ethyl pyruvate: a novel treatment for sepsis and shock. *Minerva Anestesiol*. 70: 365-371, 2004.
23. Gabbiai, F.B., Boggiano, C., Peter, T., Khang, S., Archer, C., Gold, D.P. and Kelly, C.J. Inhibition of inducible nitric oxide synthase intensifies injury and functional deterioration in autoimmune interstitial nephritis. *J. Immunol.* 159: 6266-6675, 1997.
24. Geeraerts, M.D., Ronveaux-Dupal, M.F., Lemasters, J.J. and Herman, B. Cytosolic free Ca^{2+} and proteolysis in lethal oxidative injury in endothelial cells. *Am. J. Physiol*. 261: C-889-C896, 1991.
25. Gurova, K.V., Kwek, S.S., Koman, I.E., Komarov, A.P., Kandel, E., Nikiforov, M.A. and Gudkov, A.V. Apoptosis inhibitor as a suppressor of tumor progression: expression of Bcl-2 eliminates selective advantages for p53-dificient cells in the tumor. *Cancer Biol. Ther*. 1: 39-44, 2002.
26. Halliwell, B. and Gutteridge, J. Free Radicals in Biology and Medicine, Oxford University Press, Oxford, 1999.
27. Hierholzer, C. and Billiar, T.R. Molecular mechanism in the early phase of hemorrhagic shock. *Langenbecks Arch. Surg*. 386: 302-308, 2001
28. Hierholzer, C., Harbrecht, B., Menezes, J.M., Kane, J., MacMicking, J. and Nathan, C.F. Essential role of induced nitric oxide in the initiation of the inflammatory responses after hemorrhagic shock. *J. Exp. Med.* 187: 917-928, 1998.
29. Hill, M.M., Adrain, C. and Martin, S.J. Portrait of a killer: The mitochondrial apoptosome emerges from the shadow. *Mol. Intenventions* 3: 19-25, 2003.
30. Irvine, R.A., Adachi, N., Shibata, D.K., Cassell, G.D., Yu, K., Karanjawala, Z.E., Hsieh, C.L. and Lieber, M.R. Generation and characterization of endonuclease G null mice. *Mol. Cell Biol*. 25: 294-302, 2005.
31. Jiang, X. and Wang, X. Cytochrome-c-mediated apoptosis. *Annu. Rev. Biochem*. 73: 87-106, 2004.
32. Johnson, J.D., Conroy, W.G. and Isom G.E. Alteration of cytosolic calcium levels in PC12 cells by potassium cyanide. *Toxicol. Appl. Pharmacol*. 88: 217-224, 1987.
33. Kappeler, L. and Epelbaum, J. Biological aspects of longevity and ageing. *Rev. Epidemiol. Sante Publique.* 53: 235-241, 2005.
34. Kiang, J.G. Effect of intracellular pH on cytosolic free $[Ca^{2+}]_i$ in A-431 cells. *Eur. J. Pharmacol. 207(Molecular Pharmacol. Section)*: 287-296, 1991.
35. Kiang, J.G. Inducible heat shock protein 70 kD and inducible nitric oxide synthase in hemorrhage/resuscitation-induced injury. *Cell Res.* 14: 450-459, 2004.
36. Kiang, J.G., Bowman, P.D., Lu, X., Li, Y., Ding, X.Z., Zhao, B., Juang, Y.T., Atkins, J.L. and Tsokos, G.C. Geldanamycin prevents hemorrhage-induced ATP loss by overexpressing inducible HSP70 and activating pyruvate dehydrogenase. *Am. J. Physiol. (Gastrointest. Liver Physiol.)* 291: G117-G127, 2006.
37. Kiang, J.G., Bowman, D.P., Wu, B.W., Hampton, N., Kiang, A.G., Zhao, B., Juang, Y.-T., Atkins, J.L. and Tsokos, G.C. Geldanmaycin inhibits hemorrhage-induced increases in caspase-3 activity, KLF6, and iNOS expression in unresuscitated organs of mice: Role of inducible HSP-70. *J. Appl. Phsiol*. 97: 564-569, 2004.
38. Kiang, J.G., Bowman, P.D., Wu, B.W., Hampton, N., Kiang, A.G., Zhao, B., Juang, Y.-T., Atkins, J.L. and Tsokos, G.C. Geldanamycin inhibits hemorrhage-induced increases in caspase-3 activity, KLF6 and iNOS expression in unresuscitated organs of mice: Role of inducible HSP-70. *FASEB J*. 18: A220-A221, 2004.
39. Kiang, J.G., Ding, X.Z. and McClain, D.E. Overexpression of HSP-70 attenuates increases in $[Ca^{2+}]_i$ and protects human epidermoid A-431 cells after chemical hypoxia. *Toxicol. Appl. Pharmacol*. 149: 185-194, 1998.
40. Kiang, J.G., Kiang, S.C., Juang, Y.-T. and Tsokos, G.C. N^{ω}-nitro-L-arginine inhibits inducible HSP-70 *via* Ca^{2+}, PKC, and PKA in human intestinal epithelial T84 cells. *Am. J. Physiol*. 282: G415-G423, 2002.
41. Kiang, J.G., Lu, X., Tabaku, L.S., Bentley, T.B., Atkins, J.L. and Tsokos, G.C. Resuscitation with lactated Ringer solution limits the expression of molecular events associated with lung injury after hemorrhage. *J. Appl. Physiol*. 98: 550-556, 2005.
42. Kiang, J.G., Peckham, R.M. Duke, L.E., Shimizu, T., Chaudry, I.H. and Tsokos, G.C. Androstenediol inhibits the trauma-hemorrhage-induced increase in caspase-3 by downregulating the inducible nitric oxide synthase pathway. *J. Appl. Physiol*. 2006 (in review).
43. Kiang, J.G. and Smallridge, R.C. Sodium cyanide increases cytosolic free calcium: evidence for activation of the reversed mode of the Na^+/Ca^{2+} exchanger and Ca^{2+} mobilization from inositol trisphosphate-insensitive pools. *Toxicol. Appl. Pharmacol*. 127: 173-181, 1994.
44. Kiang, J.G. and Tsokos, G.C. Heat shock protein 70 kDa: Molecular Biology, Biochemistry, and Physiology. *Pharmacol. Ther*. 80: 183-201, 1998.
45. Kiang, J.G. and Tsokos, G.C. Signal transduction and heat shock protein expression. *J. Biomed. Sci*. 3: 379-388, 1996.
46. Kiang, J.G., Wang, X.D., Ding, X.Z., Gist, I. and Smallridge, R.C. Heat shock inhibits the hypoxia-induced effects on iodide uptake and signal transduction and enhances cell survival in rat thyroid FRTL-5 cells. *Thyroid* 6: 475-483, 1996.
47. Kiang, J.G., Warke, V.G. and Tsokos, G.C. NaCN-induced chemical hypoxia is associated with altered gene expression. *Mol. Cell. Biochem*. 254: 211-216, 2003.
48. Kline, J.A., Maiorano, P.C., Schroeder, J.D., Grattan, R.M., Vary, T.C. and Watts, J.A. Activation of pyruvate dehydrogenase improves heart function and metabolism after hemorrhagic shock. *J. Mol. Cell Cardiol*. 29: 2465-2474, 1997.
49. Lee, H.H., Kim, H., Lee, M.H., Chang, H.K., Lee, T.H., Jang, M.H., Shin, M.C., Lim, B.V., Shin, M.S., Kim, Y.P., Yoon, J.H., Jeong, I.G. and Kim, C.J. Treadmill exercise decreases intrastriatal hemorrhage-induced neuronal cell death *via* suppression on caspase-3 expression in rats. *Neurosci. Lett*. 352: 33-36, 2003.
50. Leist, M., Single, B. and Castoldi, A.F. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. *J. Exp. Med*. 185: 1481-1486, 1997.
51. Lemasters, J.J. Mechanisms of hepatic toxicity: V-necrapoptosis and the mitochondrial permeability transition: shared pathways for necrosis and apoptosis. *Am. J. Physiol*. 276: G1-G6, 1999.
52. Liberthal, W., Menza, S.A. and Levine, J.S. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. *Am. J. Physiol*. 274: F314-F327, 1998.
53. Luo, Y. Long-lived worms and aging. *Redox. Rep*. 9: 65-69, 2004.
54. Maduh, E.U., Borowitz, J.L. and Isom, G.E. Cyanide-induced alteration of cytosolic pH: Involvemnt of cellular hydrogen ion handling processes. *Toxicol. Appl. Pharmacol*. 106: 201-208, 1990.
55. Marsden, V.S., O'Connor, L., O'Reilly, L.A., Silke, J., Metcalf, D., Ekert, P.G., Huang, D.C., Cecconi, F., Kuida, K., Tomaselli, K.J., Roy. S., Nicholson, D.W., Vaux, D.L., Bouillet, P., Adams, J.M. and Strasser, A. Apoptosis initiated by Bcl-2-regulated caspase activation independently of the cytochrome c/Apaf-1/caspase-9 apoptosome. *Nature* 419: 634-637, 2002.
56. Mauriz, J.L., Gonzalez, P., Jorquera, F., Olcoz, J.L. and Gonzalez-Gallego. J. Caspase inhibition does not protect against liver damage in hemorrhagic shock. *Shock* 19: 33-37, 2003.
57. Meldolesi, J. and Pozzan, T. Pathways of Ca^{2+} influx at the plasma membrane: Voltage-, receptor-, and second messenger-operated channels. *Exp. Cell Res*. 171: 271-278, 1987.

58. Mizushima, Y., Wang, P., Jarrar, D., Cioffi, W.G., Bland, K.I. and Chaudry, I.H. Preinduction of heat shock proteins protects cardiac and hepatic functions following trauma and hemorrhage. *Am. J. Physiol. (Regul. Integr. Comp. Physiol*.) 278: R352-R359, 2000.
59. Mongan, P.D., Capacchione, J., West, S., Karaian, J., Dubois, D., Keneally, R. and Sharma, P. Pyruvate improves redox status and decreases indicators of hepatic apoptosis during hemorrhagic shock in swine. *Am. J. Physiol. (Heart Circ. Physiol.)* 283: H1634-1644, 2002.
60. Moore, W.M., Webber, R.K., Jerome, G.M., Tjoeng, F.S., Misko, T.P. and Currie, M.G. L-N^6-(1-iminoethyl)lysine: a selective inhibitor of inducible nitric oxide synthase. *J. Med. Chem*. 37: 3886-3888, 1994.
61. Murao, Y., Hata, M., Ohnishi, K., Okuchi, K., Nakajima, Y., Hiasa, Y., Junger, W.G., Hoyt, D.B. and Ohnishi, T. Hypertonic saline resuscitation reduces apoptosis and tissue damage of the small intestine in a mouse model of hemorrhagic shock. *Shock* 20: 23-28, 2003.
62. Nagoshi, T., Matsui, T., Aoyama, T., Leri, A., Anversa, P., Li, L., Ogawa, W., del Monte, F., Gwathmey, J.K., Grazette, L., Hemmings, B.A., Kass, D.A., Champion, H.C. and Rosenzweig, A. PI3K rescues the detrimental effects of chronic Akt activation in the heart during ischemia/reperfusion injury. *J. Clin. Investig*. 115: 2128-2138, 2005.
63. Nakada, H., Kase, Y., Matsunaga, T., Komoda, T. and Iinuma, T. Caspase 3 activation in nasal capillary in patients with epistaxis. *Otolaryngol. Head Neck Surg*. 128: 632-639, 2003.
64. Nalos, M., Asfar, P., Ichai, C., Radermacher, P. and Leverve, X.M. Adenosine trisphosphate-magnesium chloride: Relevance for intensive care. *Int. Care Med*. 29: 10-18, 2003.
65. Niki, I., Yokokura, H., Sudo, T., Kato, M. and Hidaka, H. Ca^{2+} signaling and intracellular Ca^{2+} binding proteins. *J. Biochem*. 120: 4685-4698, 1996.
66. Noma, K., Oyama, N. and Liao, J.K. Physiological role of ROCKs in the cardiovascular system. *Am. J. Physiol. (Cell Physiol.)* 290: C661-C668, 2006.
67. O'Mahoney, M.E., Logue, S., Szegezdi, E., Stenson-Cox, C., Fitzgerald, U. and Samali, A. Hypoxia and ischemia induce nuclear condensation and caspase activation in cardiomyocytes. *Ann. N.Y. Acad. Sci.* 1010: 728-732, 2003.
68. Orrenius, S., McConkey, D.J., Bellomo, G. and Nicotera, P. Role of Ca^{2+} in toxic cell killing. *Trends Pharmacol. Sci*. 10: 281-285, 1989.
69. Paxian, M., Bauer, I., Rensing, H., Jaeschke, H., Mautes, A.E., Kolb, S.A., Wolf, B., Stockhausen, A., Jeblick, S. and Bauer, M. Recovery of hepatocellular ATP and "pericentral apoptosis" after hemorrhage and resuscitation. *FASEB J*. 17: 993-1002, 2003.
70. Pearce, F.J., Connett, R.J. and Drucker, W.R. Phase-related changes in tissue energy reserves during hemorrhagic shock. *J. Surg. Res.* 39: 390-398, 1985.
71. Pelacho, B., Natal, C., Espana, A., Sanchez-Carpintero, I., Iraburu, M.J. and Lopez-Zabalza, M.J. Pemphigus vulgaris autoantibodies induce apoptosis in HaCaT keratinocytes. *FEBS Lett.* 566: 6-10, 2004.
72. Philosoph, H. and Zilberstein, D. Regulation of intracellular calcium in promastigotes of the human protozoan parasite Leishmania donovani. *J. Biol. Chem*. 264: 10420-10424, 1989.
73. Pittet, J.F., Lu, L.N., Geiser, T., Lee, H., Matthay, M.A. and Welch, W.J. Stress preconditioning attenuates oxidative injury to the alveolar epithelium of the lung following haemorrhage in rats. *J. Physiol*. 538: 583-597, 2001.
74. Portelli, C. The role of the sodium, potassium, magnesium and calcium ions in the transfer of energy at the level of the ATP molecule. *Physiologie* 13: 315-319, 1976.
75. Saika, M., Ueyama, T. and Senba, E. Expression of immediate early genes, HSP70, and COX-2 mRNAs in rat stomach following ethanol ingestion. *Dig. Dis. Sci.* 45: 2455-2462, 2000.
76. Sakaida, I., Thomas, A.P. and Farber, J.L. Phospholipid metabolism and intracellular Ca^{2+} homeostasis in cultured rat hepatocytes intoxicated with cyanide. *Am. J. Physiol*. 263: C684-C690, 1992.
77. Salerno, L., Sorrenti, V., Di Giacomo, C., Romeo, G. and Siracusa, M.A. Progress in the development of selective nitric oxide synthase (NOS) inhibitor. *Curr. Pharm. Design* 8: 177-200, 2002.
78. Sato, M., Maulik, N. and Das, D.K. Cardioprotection with alcohol: role of both alcohol and polyphenolic antioxidants. *Ann. N. Y. Acad. Sci*. 957: 122-135, 2002.
79. Sato, M., Ikeda, K., Yoshizaki, K. and Koyano, H. Responses of cytosolic calcium to anoxia and cyanide in cultured glomus cells of newborn rabbit carotid body. *Brain Res.* 551: 327-330, 1991.
80. Sebbagh, M., Renvoize, C., Hamelin, J., Riche, N., Bertoglio, J. and Breard, J. Caspase-3-mediated cleavage of ROCK 1 induces MLC phosphorylation and apoptotic membrane blebbing. *Nature Cell Biol*. 3: 346-352, 2001.
81. Shea-Donohue, T., Anderson, J. and Swiecki, C. Ischemia/reperfusion injury. In: Combat Medicine - Basic and clinical research in military, trauma, and emergency medicine (eds G.C. Tsokos and J.L. Atkins), Humana Press, New Jersey, 2003 pp. 219-248.
82. Shimizu, T., Szalay, L., Choudhry, M.A., Schwacha, M.G., Rue, L.W. III, Bland, K.I. and Chaudry, I.H. Mechanism of salutary effects of androstenediol on hepatic function following trauma-hemorrhage: the role of endothelin 1 and inducible nitric oxide synthase. *Am. J. Physiol*. 288: G244-G250, 2005.
83. Slomiany, B.L., Piotrowski, J. and Slomiany, A. Induction of caspase-3 and nitric oxide synthase-2 during gastric mucosal inflammatory reaction to Helicobacter pylori lipopolysaccharide. *Biochem. Mol. Biol. Int*. 46: 1063-1070, 1998.
84. Smith, M.T. Role of reactive oxygen species and lipid peroxidation in chemically induced toxicity and carcinogen. In: Reactive Oxygen Species in Chemistry, Biology, and Medicine (ed. A. Quintanilha). NATO ASI Series, Plenum Press, New York, pp. 157-166, 1988.
85. Snyder, J.W., Pastorino, J.G., Thomas, A.P., Hoek, J.B. and Farber, J.L. ATP synthase activity is required for fructose to protect cultured hepatocytes from the toxicity of cyanide. *Am. J. Physiol.* 264: C709-C714, 1993.
86. Stojadinovic, A., Kiang, J.G., Smallridge, R.C., Galloway, R.L. and Shea-Donohue, T. Induction of heat-shock protein 72 protects against ischemia/reperfusion in rat small intestine. *Gastroenterology* 109: 505-515, 1995.
87. Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Contantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature* 397: 441-446, 1999.
88. Susin, S.A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Ddaugas, E., Geuskens, M. and Kroemer, G. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J. Exp. Med*. 184: 1331-1341, 1996.
89. Tavaf-Motamen, H., Miner, T.J., Starnes, B.W. and Shea-Donohue, T. Nitric oxide mediates acute lung injury by modulation of inflammation. *J. Surg. Res*. 78: 137-145, 1998.
90. Teti, A., Blair, H.C., Schlesinger, P. and Grano, M. Extracellular protons acidify osteoclasts, reduce cytosolic calcium, and promote expression of cell-matrix attachment structures. *J. Clin. Invest*. 84: 773-780, 1989.
91. Thomas, D.D., Miranda, K.M., Citrin, D., Espey, M.G. and Wink, D.A. Nittric oxide. In: Combat Medicine – Basic and clinical research in military, trauma, and emergency medicine (eds G.C. Tsokos and J.L. Atkins), Humana Press, New Jersey, pp. 23-60, 2003.
92. Trump, B.F., Berezesky, I.K., Smith, M.W., Phelps, P.C. and Elliget, K.A. The relationship between cellular ion deregulation

and acute and chronic toxicity. *Toxicol. Appl. Pharmacol*. 97: 6-12, 1989.

93. Tsen, K.T., Tsen, S.W.D. and Kiang, J.G. Lycopene is more potent than beta carotene in the neutralization of singlet oxygen: Role of energy transfer probed by ultrafast Raman spectroscopy. *J. Biomed. Optics*, 2006 (in press)
94. Tsien, R.W. Calcium channels, stores, and oscillations. *Annu. Rev. Cell Biol*. 6: 715-760, 1990.
95. Tunici, P., Schiaffonati, L., Rabellotti, E., Tiberio, L., Perin, A. and Sessa, A. *In vivo* modulation of 73 kDa heat shock cognate and 78 kDa glucose-regulating protein gene expression in rat liver and brain by ethanol. *Alcohol Clin. Exp. Res*. 23: 1861-1867, 1999.
96. Van Way, C.W. 3rd, Dhar, A., Morrison, D.C., Longorio, M.A. and Maxfield, D.M. Cellular energetics in hemorrhagic shock: restoring adenosine triphosphate to the cells. *J. Trauma 54 (Suppl)*: S169-S176, 2003.
97. Vaux, D., Weissman, I.L. and Kim, S.K. Prevention of programmed cell death in *C. elegans* by human bcl-2. *Science* 258: 1955-1957, 1992.
98. Wang, X., Mori, T., Sumii, T. and Lo, E.H. Hemoglobin-induced cytotoxicity in rat cerebral cortical neurons: Caspase activation and oxidative stress. *Stroke* 33: 1882-1888, 2002.
99. Wang, X., Zhu, C., Wang, X., Hagberg, H., Korhonen, L., Sandberg, M., Lindholm, D. and Blomgren, K. X-linked inhibitor of apoptosis (XIAP) protein protects against caspase activation and tissue loss after neonatal hypoxia-ischemia. *Neurobiol. Dis*. 16: 179-189, 2004.
100. Wang, X., Yang, C., Chai, J., Shi, Y. and Xue, D. Mechanisms of AIF-mediated apoptotic DNA degradation in *Caenorhabditis elegans*. *Science* 298: 1587-1592, 2002.
101. Wiegele, G., Brandis, M. and Zimmerhackl, L.B. Apoptosis and necrosis during ischemia in renal tubular cells (LLC-PK and MDCK). *Nephrol. Dial. Transplant.* 13: 1158-1167, 1998.
102. Yu, H.P., Hsieh, Y.C., Suzuki, T., Shimizu, T., Choudhry, M.A., Schwacha, M.G. and Chaudry, I.H. Salutary effects of estrogen receptor-beta agonist on lung injury after trauma-hemorrhage. *Am. J. Physiol.* 290: L1004-L1009, 2006.
103. Yu, J.L., Rak, J.W., Coomber, B.L., Hicklin, D.J. and Kerbel, R.S. Effect of p53 status on tumor response to antiangiogenic therapy. *Science* 295: 1526-1528, 2002.
104. Zhou, C., Yamaguchi, M., Kusaka, G., Schonholz, C., Nanda, A. and Zhang, J.H. Caspase inhibitors prevent endothelial apoptosis and cerebral vasospasm in dog model of experimental subarachnoid hemorrhage. *J. Cerebr. Blood F. Met.* 24: 419-431, 2004.
105. Zhu, Y., Mao, X.O., Sun, Y., Xia, Z. and Greenberg, D.A. p38 Mitogen-activated protein kinase mediates hypoxic regulation of Mdm2 and p53 in neurons. *J. Biol. Chem*. 277: 22909-22914, 2002.
106. Zubrow, A.B., Delivoria-Papadopoulos, M., Ashraf, Q.M., Ballesteros, J.R., Fritz, K.I. and Mishra, O.P. Nitric oxide-mediated expression of Bax protein and DNA fragmentation during hypoxia in neuronal nuclei from newborn piglets. *Brain Res*. 954: 60-67, 2002.