

NITRIC OXIDE IN THE LIVER

MARK G. CLEMENS

CHEMISTRY AND BIOCHEMISTRY OF NITRIC OXIDE AND NITRIC OXIDE SYNTHASES 555

Chemistry of Nitric Oxide 555

Enzymatic Production on Nitric Oxide 556

REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY 557

Transcriptional Regulation 557

Posttranslational Regulation 557

NITRIC OXIDE IN NORMAL REGULATION 557

Blood Flow Regulation 557

Carbon Monoxide in Hepatic Regulation 559

REGULATION OF METABOLISM 560

NITRIC OXIDE IN APOPTOSIS 561

Nitric oxide (NO) is a pluripotent gaseous free radical that has been identified as an important signaling molecule in virtually every tissue in the body. In the liver, like many other organs, NO has many actions and can be derived from multiple cellular sources. As a result, the exact role of NO in regulating cell or organ function is complex, and experimental evidence often appears to be contradictory. Moreover, the wealth of information concerning the role of NO in the liver is growing at a rapid rate. Prior to 1994, when the previous edition of this book was published, Medline listed 4,731 publications on NO, of which 205 matched for *nitric oxide* and *liver*. In 2000, Medline listed 33,556 publications on NO, with 1,584 of them matching for *nitric oxide* and *liver*. In spite of the proliferation of published studies, the exact role of NO in biologic regulation remains controversial. Because of the liver's complex complement of cell types, all of which are likely to be important sources of NO, the role of NO in the liver can be particularly confusing. Nitric oxide is clearly involved in normal regulation of liver function; moreover, a selective review of the literature can result in compelling evidence that NO is a primary mediator of liver cell injury. An equally compelling case can be made for the hypothesis that NO generation constitutes a potent protective mechanism in the face of potentially injurious stimuli. These apparently discrepant findings appear to be largely the result of diverse effects of NO depending on the microenvironment in which it is generated as well as the variable activity of the nitric oxide synthases. These enzymes can produce either nitric oxide or

superoxide under different conditions of availability of substrate and cofactors. Since this chapter specifically summarizes the role of NO in the liver, an exhaustive treatment of the basic chemistry and biochemistry of NO and the nitric oxide synthases is not presented. Nevertheless, some basic properties of both NO and the enzymes that generate it are pertinent to the understanding of its role in regulating hepatic function and will be summarized to provide a context for the discussion of the role of NO in regulating liver function.

CHEMISTRY AND BIOCHEMISTRY OF NITRIC OXIDE AND NITRIC OXIDE SYNTHASES

NO is a gaseous radical, the majority of which is produced by a family of enzymes, the nitric oxide synthases (NOSs). All three isoforms of NOS are found in the liver (1–5). Of these, the inducible (inflammatory) NOS (iNOS, NOS-2) and the endothelial constitutive (eNOS, NOS-3) are the most important. The neuronal constitutive (nNOS, NOS-1) form appears to be restricted to nerve endings found in the larger blood vessels, and the functional implications of this isoform remain to be elucidated (2). Thus, this chapter focuses on the role of NO produced by eNOS and iNOS, with the recognition that future work may identify an important function for nNOS in the liver.

Chemistry of Nitric Oxide

Somewhat paradoxically, the very simple chemical structure of NO constituted a major barrier to the elucidation of its

M. G. Clemens: Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223.

very important function as an important endogenous biologic regulator. It was unprecedented that such a simple, carbonless gas produced by an enzymatic reaction could exert such important signaling functions. This is especially true in the context that the vast majority of signaling functions are mediated by noncovalent binding based on specific molecular shape properties. NO, on the other hand, interacts with target molecules via covalent redox reactions (6). In spite of its chemical simplicity, its specific reactivity with biologic molecules confers upon NO the potential to regulate cellular function at multiple levels. Moreover, the simplicity of NO is overcome by the complex regulation of the enzymes responsible for its synthesis.

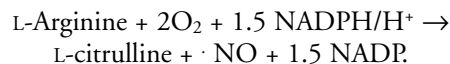
Probably the most important of the reactions of NO from the point of view of biologic regulation is the reaction with iron in heme proteins to form nitrosyl complexes (7–9). This reaction can be an important modulator of the function of the heme proteins. On the one hand, such an interaction with a heme protein can cause activation. A common example is in the activation of guanyl cyclase by NO. NO binding results in the activation of the enzyme and 3',5'-cyclic guanosine monophosphate (cGMP) production. On the other hand, NO binding to mitochondrial aconitase results in inactivation of enzyme activity. Alternatively, NO binding to hemoglobin results in either scavenging (inactivation) of NO, thus limiting the biologic half-life of NO, or transport of NO to remote tissues where it can be released and exert biologic actions (9). The significance of these binding properties for hepatic regulation is described below. Finally, NO may serve to reduce heme iron in the Fe^{4+} state to Fe^{3+} . In doing so, NO can limit the oxidizing potential of pro-oxidant iron (6). Thus, the reactions of NO with iron proteins alone are complex, with the functional result depending on the protein.

Second to its ability to react with metal complexes in proteins, the reaction of NO with other radicals, especially superoxide (O_2^-), is likely to be the most important (10,11) (see Chapters 18 and 19 and website chapter [W-13](#)). The significance of this reaction is also complicated. On the one hand, reaction with O_2^- has been proposed to be a major mechanism of the antiinflammatory action of NO in

that it scavenges superoxide. On the other hand, the product of this reaction, peroxynitrite (ONOO^-) is potentially more toxic than either of its precursors. Finally, NO can react directly with thiol groups or, following generation of ONOO^- , with tyrosine hydroxyl groups on cellular enzymes (8). In either case, these interactions typically result in inhibition of enzyme activity.

Enzymatic Production on Nitric Oxide

The vast majority of NO produced in biologic systems is the result of the enzymatic conversion of L-arginine to L-citrulline by NOSs. Although the different isozymes are encoded by different genes located on different chromosomes, there is considerable sequence homology in the NOS proteins (12). Most notably, several regions essential for catalytic activity are highly conserved. These include binding sites for reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and calmodulin, as well as dependence on tetrahydrobiopterin (BH_4) as a cofactor. As such, all three forms consume oxygen and receive electrons from NADPH, which are then transferred to L-arginine to form NO plus L-citrulline:



This reaction involves the formation of several intermediates resulting from sequential electron transfer steps. It is significant that the transfer of the electron from NADPH to O_2 is fairly independent of BH_4 or substrate (L-arginine) availability, while the completion of the transfer of electrons to L-arginine is highly dependent on the presence of adequate BH_4 and L-arginine (13–16) (Fig. 39.1). This property of the NOS enzymes has potentially very important implications for modulation of liver function since the reduction of O_2 by NADPH generates O_2^- (superoxide). Thus in the absence of sufficient L-arginine or BH_4 , activated NOS will produce O_2^- rather than NO. In reality, it is unlikely that, under *in vivo* conditions, NOS will completely switch over from an NO to an O_2^- generating system. Instead, cogeneration of

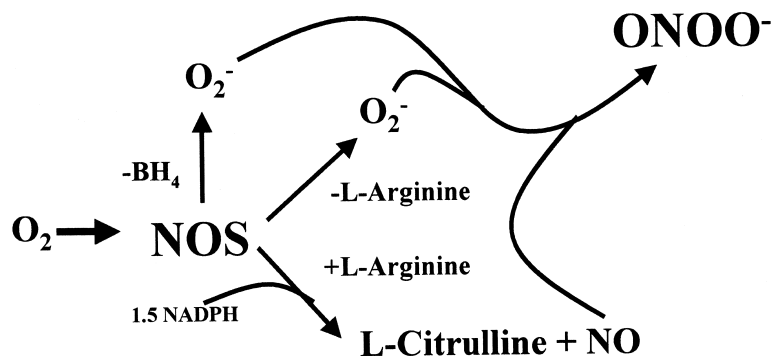


FIGURE 39.1. Generation of superoxide and peroxynitrite by nitric oxide (NO) synthases (NOS) in conditions of substrate or cofactor limitation. (Based on refs. 12, 14, and 15.)

NO and O_2^- is the likely result. As described above, the combination of NO and O_2^- results in the formation of peroxynitrite, which can be extremely toxic.

REGULATION OF NITRIC OXIDE SYNTHASE ACTIVITY

Transcriptional Regulation

Although iNOS and eNOS are commonly differentiated as inducible and constitutive forms, respectively, both forms are subject to regulation at the level of gene expression. Regulation by gene expression is most important for iNOS, as it constitutes the principal mechanism for regulation. Originally identified as being induced in macrophages, iNOS is now known to be induced in a wide range of cells. In the liver these include not only the Kupffer cells, but also hepatocytes, vascular endothelial cells, smooth muscle cells, and hepatic stellate cells. In general, iNOS is induced by inflammatory mediators (17). While iNOS can be induced by individual mediators, the level of induction is synergistically affected by combinations of extracellular stimuli such as the so-called cytomix [tumor necrosis factor- α (TNF- α) + interleukin-1 β (IL-1 β) + interferon- γ (IFN- γ) + endotoxin]. This synergism reflects the complexity of the promoter region of the human iNOS gene. Interestingly, in spite of the homology of NOS proteins between species, the iNOS promoter shows substantial variation between the human and murine genes (17). The human iNOS gene has a 5' flanking region of approximately 16 kilobases (kb) and is dependent primarily on the binding of nuclear factor κ B (NF κ B), activating protein (AP-1), and signal transducer and activator of transcription 1 α (STAT-1 α) to their respective consensus sequences. In contrast, the murine 5' flanking region is approximately 1 kb yet it contains additional sequences for IFN-stimulated response elements (ISREs) and hypoxia response elements (HREs). These differences may account for some of the differential results reported in studies in mice versus those using human cells. Nevertheless, in both species, activation of gene transcription by signaling pathways associated with inflammation is the primary mechanism for positive regulation of iNOS activity.

It is noteworthy that inflammatory mediators such as endotoxin and cytokines constitute far more potent inducers of the iNOS gene than does oxidative stress such as is associated with ischemia or reperfusion. iNOS transcription is also inhibited by transforming growth factor- β (TGF- β), which further contributes to decreased gene expression by destabilizing iNOS messenger RNA (mRNA).

Although eNOS is commonly considered to be a constitutive enzyme that is regulated posttranslationally by Ca^{2+} and calmodulin, enzyme levels are also regulated. Levels of eNOS are upregulated by stimuli such as shear stress (18). Levels are also upregulated in response to certain cholesterol-lowering drugs such as simvastatin (19). Conversely,

eNOS levels have been reported to be decreased during inflammatory states such as endotoxemia (20,21).

Posttranslational Regulation

Unlike iNOS, which is primarily regulated by altering the amount of enzyme present, eNOS is exquisitely sensitive to posttranslational regulation (22). This degree of regulation is the result of the nature of the interaction between the NOS enzyme and calmodulin. While iNOS binds calmodulin and thus is active even at Ca^{2+} concentrations below that in resting cells, eNOS requires that calmodulin be bound to Ca^{2+} before binding. As a result, eNOS activity is primarily regulated by fluctuations in intracellular Ca^{2+} . This Ca^{2+} dependence allows eNOS to be regulated with a very short time constant and to be responsive to the presence of endothelium-dependent vasodilators [e.g., acetylcholine or adenosine triphosphate (ATP)] as well as physical factors such as shear stress.

Recent studies have also demonstrated that binding of calmodulin to eNOS can also be regulated by interactions with membrane-associated proteins such as caveolin-1 (22,23). eNOS is found in the particulate fraction of tissue homogenates, indicating that it normally exists as a membrane bound protein. Immunoprecipitation studies indicate that eNOS is associated with the specific membrane subdomain of the caveolae, where it is bound to caveolin-1. Binding to caveolin-1 maintains the eNOS in an inactive state until it is displaced by Ca^{2+} calmodulin, at which point it moves to the cytoplasm in an active state and generates NO. The significance of this interaction is that overexpression of caveolin-1 has been reported in conditions such as cirrhosis (24). Moreover, increased expression of caveolin-1 decreases the interaction of eNOS with calmodulin. This level of regulation may contribute significantly to the microvascular deficits observed in cirrhosis (see Blood Flow Regulation, below).

The association of eNOS with caveolae is related to its posttranslational regulation by fatty acylation (25). This involves the addition of two molecules of myristic acid during translation. This step is thought to be irreversible and is necessary for targeting to the caveolar membrane domain. An additional site can also be palmitoylated in a reversible manner. Both types of acylation serve to help anchor the NOS enzyme in the membrane, where it is held in an inactive state bound to caveolin. Palmitoylation is sensitive to signaling molecules and may serve as an additional mechanism to regulate the release of the eNOS from the membrane during agonist stimulation (22).

NITRIC OXIDE IN NORMAL REGULATION

Blood Flow Regulation

The significance of NO as an endogenous biologic signaling molecule was originally recognized in the context of its

role as a vasodilator serving as the endothelium-dependent relaxing factor (26). In this capacity, NO is well recognized as an important regulator of local blood flow in many vascular beds. The role of NO in regulation of the hepatic circulation, however, has been less clear. Although a role for NO in the control of basal resistance in the hepatic artery has been easily identified (27–29), a functional role for eNOS in the portal circulation has been more controversial (30–32). On the other hand, iNOS is virtually absent in the normal liver, but highly upregulated in response to a variety of inflammatory or oxidative stresses. This led to the common postulate that iNOS, but not eNOS, contributed to maintaining sinusoidal perfusion following stress conditions (32). While very logical, the experimental results suggest that the situation is more complicated. Treatment of rats with nonspecific NOS or primarily eNOS inhibitors results in a rapid exacerbation of injury following stresses such as endotoxin injection (33,34). This exacerbation of injury is associated with local failure of microvascular perfusion (32) and the development of patchy necrosis (33). At the same time, in spite of marked upregulation of iNOS under these conditions, specific iNOS antagonists have little effect on liver perfusion and typically result in amelioration of injury if any effect is observed (35,36). Improvement in organ function appears to be more a function of support of the systemic circulation than of a direct effect on liver function (37). These results suggest that NO is necessary to maintain the sinusoidal perfusion, but that the relatively small amount generated by eNOS is adequate. The specific compartmentalization in the endothelial cells may also be significant in maintaining perfusion in that NO generated by endothelial cells or hepatic stellate cells, even at low levels, is capable of diffusing to the sites of action for vasoregulation (38).

Altered activity of eNOS may also be important in the development of increased intrahepatic resistance during the development of injury that leads to cirrhosis. Sinusoidal endothelial cells isolated from livers of rats subjected to CCl₄ or bile duct ligation exhibit a specific decrease in the enzymatic activity of eNOS without a decrease in total eNOS protein expression (4). A probable mechanism for this apparent posttranslational event that serves to functionally downregulate eNOS activity has recently been reported (24). As described above, eNOS is normally localized in caveolar subdomains of the plasma membrane of endothelial cells where it is found bound to the membrane protein caveolin-1 (22). A similar localization of eNOS associated with caveolae has been reported in cardiac myocytes in which eNOS binds to caveolin-3. There are two characteristics of this interaction that are of major importance: (a) binding of caveolin and Ca²⁺-calmodulin by eNOS are mutually exclusive, and (b) binding of eNOS to caveolin maintains the eNOS in an inactive state. The mechanism of this inhibition appears to be the inhibition of the eNOS reductase domain by caveolin-binding (38a).

This mechanism may be highly significant in that caveolin binding inhibits not only the generation of NO but also the acceptance of electrons from NADPH. This would serve to prevent the caveolin-bound eNOS from synthesizing O₂⁻. It is also significant that the caveolin-binding domain of eNOS does not appear to be present in iNOS, although it may be present in nNOS (22). Moreover, iNOS activity does not require Ca²⁺ binding to calmodulin. Therefore, caveolin-1 upregulation is not likely to affect NO production from iNOS.

This mechanism of regulation of eNOS activity appears to be of functional significance in the liver in light of the recent report by Shah et al. (24) demonstrating that caveolin-1 expression is upregulated in livers of rats with experimental cirrhosis. What is more, even though the total amount of calmodulin found in cell lysates was not changed with cirrhosis, the amount of calmodulin that could be coprecipitated with eNOS was dramatically diminished. In contrast, the amount of caveolin-1 that coprecipitated with eNOS was substantially increased. These results would suggest that overexpression of caveolin-1 in vascular endothelial cells of cirrhotic livers serves to functionally impair eNOS activity. This interpretation is further supported by the observation that NO production was decreased in these livers and vascular resistance was increased. Interestingly, preliminary results from our lab indicate that caveolin-1 is also upregulated in endotoxemia, a condition that also leads to increased portal resistance. This observation provides at least preliminary evidence that altered regulation of caveolin may be a common mechanism for the development of vascular deficits in the liver during inflammatory states.

There is reason to believe that NO may be involved in limiting the progression of liver diseases that involve vascular alterations such as in cirrhosis. Recent work from Rockey's group (39) showed that the portal hypertension that accompanies cirrhosis can be substantially ameliorated by transient transduction of the liver with nNOS. Hepatic stellate cells relax in response to NO both *in vitro* (40) and *in situ* (31) (see Chapter 39). In Rockey's group's (39) experiments, the adenovirus vector preferentially transduced the hepatic stellate cells. As a result, local production of NO would limit the vasoconstrictive effects of stellate cell activation, thus limiting perfusion deficits. Indeed, Rockey's group found that the transduced livers had a significantly lower portal pressure. Interestingly, the major effect on resistance was on the pressure at zero flow, which is an indicator of sinusoidal sites of action. This finding is consistent with action of NO on the hepatic stellate cells to limit the resistance to flow. In addition, the antiinflammatory actions of NO may serve to limit the activation of the stellate cells. These areas warrant further investigation.

The exact mechanisms by which eNOS regulates sinusoidal perfusion is not clear. Sinusoidal endothelial cells respond to shear stress with increased NO release (5,41), but the portal circulation does not dilate in response to the

classic endothelium-dependent vasodilator ATP (42). However, endothelin acting through endothelin (ET)_{B1} receptors is coupled to NO production, while putative ET_{B2} receptors cause constriction (43). Many studies have shown that endothelin and its receptors are altered in response to injurious or inflammatory stimuli in the liver. ET_B receptors are upregulated in cirrhosis (44) and following inflammatory or oxidative stress (45). This change in receptor expression may contribute to the changes in vascular response in these stress conditions (46). Recent work from our lab has shown that ET_B receptors are upregulated during endotoxemia. Concurrently, the portal pressure and sinusoid constrictor responses to ET_B agonists as well as liver injury are markedly potentiated by pretreatment with L-NAME (47). Indeed, in the absence of L-NAME, the ET_B agonist IRL 1620 did not cause sinusoidal constriction while in the presence of L-NAME; contraction of the stellate cells resulting in constriction of the sinusoid was clearly demonstrated (47). These results suggest a compensatory upregulation of eNOS stimulation via increased coupling to ET_B receptors. Such a response would contribute to the protection of sinusoidal perfusion during upregulation of endothelins. One caveat that must be considered in interpreting results reporting the effects of NOS inhibitors on liver perfusion is that initiation of inflammation in the liver typically results in systemic effects that give rise to induction of iNOS in peripheral vascular tissue. This is certainly the case in endotoxemia or sepsis, in which the circulatory collapse that results in septic shock has been ascribed to the gross overexpression of iNOS in resistance vessels (48). As such, treatment *in vivo* with NOS inhibitors is likely to produce significant changes in hepatic perfusion just by virtue of the fact that systemic hemodynamics are disrupted.

Although NO was originally identified as a modulator of blood flow via its vasodilatory properties, it also has an important impact on injury-related blood flow regulation by virtue of its effect on neutrophil adhesion to vascular endothelium as well as platelet aggregation. Considerable evidence now indicates that neutrophil accumulation following inflammatory or oxidative stress in the liver contributes to hepatocyte injury (49,50). In such cases, administration of NOS antagonists significantly increases the accumulation of neutrophils and exacerbates liver injury to a similar extent (34,51). Interestingly, NOS inhibition in this study also inhibited the generation of peroxynitrite but still exacerbated injury (52). These results suggest that NO exerts an antiinflammatory effect by attenuating adhesion of neutrophils. The mechanism of this inhibitory effect on neutrophil adhesion is likely the result of inhibition of expression of vascular adhesion molecules p-selectin and intercellular adhesion molecule-1 (ICAM-1) (53). Of these, ICAM-1 is probably the more important since it is required for emigration of the neutrophils from the vascular space, and blocking ICAM-1 attenuates injury without decreasing sinusoidal neutrophil sequestration (54). NO may also

serve to antagonize sinusoidal constriction, thus attenuating physical trapping of neutrophils in the sinusoids (55). Additionally, NO released from endothelial cells inhibits platelet aggregation, thus further contributing to the maintenance of microvascular perfusion (33). In summary, NO, especially that derived from the eNOS localized in sinusoidal endothelial cells, is required to maintain perfusion of the hepatic microcirculation. This effect is mediated by a combination of (a) vasodilatory effects that counter the increased vasoconstrictor tone resulting from upregulation of endothelin, (b) inhibition of neutrophil adhesion or emigration, and (c) inhibition of platelet aggregation.

Carbon Monoxide in Hepatic Regulation

The role of NO in regulating microvascular perfusion in the liver is further complicated by the reports that carbon monoxide (CO) generated by heme oxygenase also contributes to the regulation of hepatic perfusion (56,57). Similar to the NOSs, heme oxygenases exist in constitutive and inducible forms. Heme oxygenase-1 (HO-1) is a stress-inducible isoform of HO also known as heat shock protein 32. It is induced by oxidative stress and heat shock as well as other potentially injurious stimuli, including NO (29,58). Heme oxygenase-2 is a constitutive enzyme that serves as an important step in the catabolism of heme from heme-containing proteins such as hemoglobin and the cytochromes. Both enzymes catalyze the conversion of heme to biliverdin and CO. Biliverdin is subsequently converted to bilirubin. Under normal conditions, HO-2 is the dominant isoform in the liver expressed primarily in hepatocytes. HO-1 is expressed at only low levels, primarily in Kupffer cells. CO, like NO, binds to heme proteins and can thus activate guanyl cyclase in a manner similar to NO. Although the binding affinity of CO for heme is much less than that of NO, the estimated concentrations in the microenvironment of the vasculature is much higher than that of NO. In certain injuries such as that which accompanies hemorrhagic shock and resuscitation, inhibition of HO-1, but not NOS, impairs sinusoidal perfusion and exacerbates injury (29). HO-1 induction has also been reported to be protective in sepsis (59). This suggests that NO and CO may act as redundant mechanisms to help protect sinusoidal perfusion following injury. The relative importance of each is most likely related to the specific injury, especially with respect to the relative degrees of upregulation of HO-1 vs. NOS.

While the generation of CO by HO-1 appears to be an important regulator of vascular tone, it is also necessary to consider that generation of CO by heme oxygenase also cogenerates antioxidant capacity in the form of the biliverdin–bilirubin system (Fig. 39.2). Although this has been considered to be a theoretical contributor to vascular protection, recent work has suggested that this property may account for the entire protective capacity as assessed by

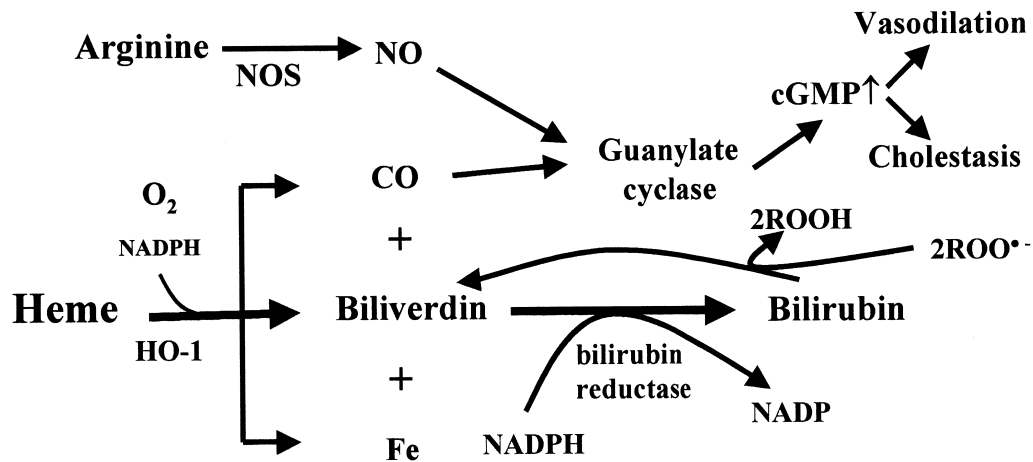


FIGURE 39.2. Mechanisms of production of carbon monoxide (CO) and biliverdin by heme oxygenase. In addition to the interaction between CO and nitric oxide (NO) in activating guanyl cyclase, the reaction produces bilirubin catalyzed by biliverdin reductase. Bilirubin is reconverted to biliverdin in scavenging reactive oxygen. The system is regenerated by biliverdin reductase in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH).

inhibition of p-selectin expression (60). This will be an important area of investigation for the near future.

In addition to its effects on the vasculature, CO has been proposed to be an important modulator of other cell functions (61). While many of these effects are likely mediated by the antioxidant properties of bilirubin, recent studies have shown that CO modulates biliary function at least partly via a cGMP-dependent mechanism. Inhibition of heme oxygenase with Zn protoporphyrin IX exerts a choleric effect that is reversed by exogenous CO and partly reversed by 8-bromo-cGMP. This effect correlates with a decrease in the spontaneous rate of bile canaliculus contraction in response to CO. Additionally, exogenous CO opens paracellular pathways between blood and bile. The functional implications of these responses are not yet well elucidated.

REGULATION OF METABOLISM

In 1985, West et al. (62) reported that conditioned medium from Kupffer cells inhibited protein synthesis in hepatocytes. This effect was subsequently found to be dependent on induction of iNOS in the hepatocytes (63). Ultimately, this discovery led to the cloning of the human iNOS from hepatocytes (64,65). Since that time, NO has been implicated in a myriad of mechanisms regulating liver metabolism. These include inhibition of protein synthesis (66,67), gluconeogenesis (68,69), and mitochondrial respiration as well as metabolic inhibition resulting from depletion of pyridine nucleotides as a result of activation of the polyadenylate ribose synthase (PARS) pathway (70). Typically, these effects require relatively high levels of NO and

may also require sufficient oxidant production (e.g., superoxide) to produce significant amounts of peroxynitrite.

It is well known that hepatic gluconeogenic response is downregulated during inflammatory states such as sepsis or endotoxemia. Moreover, the time course of changes in gluconeogenesis is similar to the appearance of iNOS induction. Exogenous NO also inhibits gluconeogenesis. The contribution of NO to the suppression of gluconeogenesis is only partial since NO specifically inhibits the activity of glyceraldehyde-3-phosphate dehydrogenase via sulfhydryl interaction (68,69) while the decrease in gluconeogenesis in sepsis is largely dependent on the transcriptional downregulation of phosphoenol pyruvate carboxykinase (71,72). This suggests that NO-mediated inhibition of gluconeogenesis may be present, but is not the primary mechanism for decreased gluconeogenesis.

The effects of NO on mitochondrial respiration are likely to be of greater significance. NO interacts with the heme groups of the cytochromes of the electron transport chain, resulting in decreased respiratory activity (73–76). The net effect of these actions is a decrease in the metabolic rate of the hepatocytes. Although it is clear that NO interaction with these enzymes causes decreased oxidative capacity of the mitochondria, it is not clear that this decreased capacity significantly contributes to development of liver injury. NO can also affect mitochondrial permeability.

Since many of the metabolic effects of NO appear to be regulated directly by peroxynitrite rather than NO itself, it is important to consider conditions in which peroxynitrite formation might be enhanced. While temporal association of NOS activity with the presence of oxidative stress (e.g., via xanthine oxidase activation) has been considered as a primary source of peroxynitrite, it is now recognized that all three iso-

forms of NOS are capable of generating superoxide instead of NO when substrate (arginine) or cofactor (tetrahydrobiopterin) are inadequately available (13). The result is cogeneration of NO and O_2^- by the same enzyme. This mechanism is likely to be particularly significant in inflammatory states in which highly upregulated levels of iNOS consume substantial quantities of both arginine and BH_4 . The exact functional implications remain to be elucidated.

NITRIC OXIDE IN APOPTOSIS

Perhaps the most significant direct cellular effect of NO with respect to hepatic injury is via its effect on apoptosis. Many reports have indicated that high levels of NO induce apoptosis in many cell types. This effect appears to be mediated primarily by the effect of peroxynitrite on increases in mitochondrial permeability either directly (77,78,78a) or through DNA damage with subsequent activation of the PARS pathway (79,80). This mitochondrial permeability transition results in the release of cytochrome *c* from the mitochondria, which constitutes a signal for apoptosis (72,78,81,82). More recently it has been recognized that NO can exert biphasic effects on apoptosis (83). In addition to being proapoptotic, studies indicate that even relatively low levels of NO can effectively inhibit apoptosis (84–86). At very low levels of NO, apoptosis is inhibited by inhibiting caspase-3–like activity. This effect appears to be mediated by S-nitrosylation of the enzyme (85,87). Moreover, even prolonged exposure to relatively high levels of NO can protect from TNF- α –induced apoptosis by inducing heat shock protein 70 (HSP 70) (86). It has been shown that apoptosis is a significant mechanism leading to hepatic cell death during inflammatory states. Since the same stimuli also induce iNOS in hepatocytes, this may constitute a self-limiting mechanism to control the rate of apoptosis. The notion that this is part of a regulated system is supported by the observation that p53, which leads to apoptosis in cells with DNA damage, also downregulates expression of iNOS (88,89). Thus cells responding to inflammatory stimuli for apoptosis but not those undergoing apoptosis in response to DNA damage can be salvaged by the effect of NO. Such a mechanism may provide protection against excessive cell death during inflammatory responses without preventing the elimination of cells with damaged DNA (88,89). It may also contribute to the role of iNOS induction in liver regeneration (89,90). Failure of this mechanism may be responsible for the proposed tumorigenic actions of NO (91–93). As is the case in the capacity for NO to regulate metabolic response in the liver, the effect of NO generation on apoptosis is likely to be modulated by the degree of coupling of the enzyme to NO production rather than generation of O_2^- . Thus, in the absence of adequate substrate or cofactor, activation of NOS would be predicted to lead to enhanced mitochondrial and DNA damage mediated by peroxynitrite

generation. The exact impact of uncoupling of NOS activity from NO synthesis *in vivo* remains to be elucidated.

CONCLUSION

NO plays important and diverse roles in the liver with the potential for both protection of the liver cells from injury as well as exacerbation of injury. The most important factors in determining whether NO will be protective or injurious are the localization of NO production, the amount of NO being produced, and the relative amounts of superoxide anion being produced in the same location as the NO. The small amounts of NO produced by eNOS in endothelial cells appear to be necessary and perhaps sufficient to maintain perfusion and to provide the necessary antiinflammatory and antithrombotic effects. Moreover, it is not likely that endothelial cell–derived NO exerts any injurious effect in the liver. Indeed, functional downregulation of eNOS by overexpression of and binding to caveolin-1 has been implicated as a probable mechanism for vascular impairment in experimental cirrhosis. Although upregulation of iNOS was originally considered to be part of the host defense by increasing the killing capacity of macrophages, iNOS function in the liver has focused largely on induction in hepatocytes. When the conditions are right for peroxynitrite generation, NO, via formation of peroxynitrite, can damage cellular components including DNA by its strong oxidizing effect. The probability for significant peroxynitrite synthesis is increased by inadequate arginine or BH_4 availability. This situation results in an electron being transferred to O_2 to form superoxide without completion of the transfer of the oxygen to arginine. In addition, NO can directly inhibit enzyme activities by interaction with sulfhydryl groups and metal-centered groups such as heme. Although these interactions result in inhibition of specific metabolic pathways, it is not clear that metabolic inhibition necessarily leads to cell injury but rather may constitute a normal regulatory mechanism. Indeed in the case of inhibition of caspase activity, enzyme inhibition results in protection from apoptotic cell death. Thus, although NO in very high concentrations induces cell injury under some conditions, the preponderance of evidence would suggest that under most conditions endogenous NO exerts protective effects in the liver.

REFERENCES

1. Knowles RG, Merrett M, Salter M, et al. Differential induction of brain, lung and liver nitric oxide synthase by endotoxin in the rat. *Biochem J* 1990;270:833–836.
2. Esteban FJ, Pedrosa JA, Jimenez A, et al. Distribution of neuronal nitric oxide synthase in the rat liver. *Neurosci Lett* 1997; 226:99–102.
3. Clemens MG. Does altered regulation of eNOS in sinusoidal endothelial cells determine increased intrahepatic resistance

- leading to portal hypertension? *Hepatology* 1998;27:1745–1747.
4. Rockey DC, Chung JJ. Reduced nitric oxide production by endothelial cells in cirrhotic rat liver: endothelial dysfunction in portal hypertension. *Gastroenterology* 1998;114:344–351.
 5. Shah V, Haddad FG, Garcia-Cardena G, et al. Liver sinusoidal endothelial cells are responsible for nitric oxide modulation of resistance in the hepatic sinusoids. *J Clin Invest* 1997;100:2923–2930.
 6. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls, and controls. *Cell* 1994;78:915–918.
 7. Grisham MB, Jour'd'Heuil D, Wink DA. Nitric oxide. I. Physiological chemistry of nitric oxide and its metabolites: implications in inflammation. *Am J Physiol* 1999;276:G315–G321.
 8. Miller MJ, Sandoval M. Nitric Oxide. III. A molecular prelude to intestinal inflammation. *Am J Physiol* 1999;276:G795–G799.
 9. Stamler JS, Singel DJ, Loscalzo J. Biochemistry of nitric oxide and its redox-activated forms [see comments]. *Science* 1992;258:1898–1902.
 10. Beckman JS, Koppenol WH. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. *Am J Physiol* 1996;271:C1424–C1437.
 11. Crow JP, Beckman JS. The importance of superoxide in nitric oxide-dependent toxicity: evidence for peroxynitrite-mediated injury. *Adv Exp Med Biol* 1996;387:147–161.
 12. Knowles RG, Moncada S. Nitric oxide synthases in mammals. *Biochem J* 1994;298:249–258.
 13. Vasquez-Vivar J, Kalyanaraman B, Martasek P, et al. Superoxide generation by endothelial nitric oxide synthase: the influence of cofactors. *Proc Natl Acad Sci USA* 1998;95:9220–9225.
 14. Vasquez-Vivar J, Hogg N, Martasek P, et al. Tetrahydrobiopterin-dependent inhibition of superoxide generation from neuronal nitric oxide synthase. *J Biol Chem* 1999;274:26736–26742.
 15. Schmidt HH, Hofmann H, Schindler U, et al. NO from NO synthase. *Proc Natl Acad Sci USA* 1996;93:14492–14497.
 16. Xia Y, Tsai AL, Berka V, et al. Superoxide generation from endothelial nitric-oxide synthase. A Ca²⁺/calmodulin-dependent and tetrahydrobiopterin regulatory process. *J Biol Chem* 1998;273:25804–25808.
 17. Taylor BS, Geller DA. Molecular regulation of the human inducible nitric oxide synthase (iNOS) gene [In Process Citation]. *Shock* 2000;13:413–424.[MEDLINE record in process 13:413–424.]
 18. Topper JN, Cai J, Falb D, et al. Identification of vascular endothelial genes differentially responsive to fluid mechanical stimuli: cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase are selectively up-regulated by steady laminar shear stress. *Proc Natl Acad Sci USA* 1996;93:10417–10422.
 19. Pruefer D, Scalia R, Lefer AM. Simvastatin inhibits leukocyte-endothelial cell interactions and protects against inflammatory processes in normocholesterolemic rats. *Arterioscler Thromb Vasc Biol* 1999;19:2894–2900.
 20. Chen K, Inoue M, Wasa M, et al. Expression of endothelial constitutive nitric oxide synthase mRNA in gastrointestinal mucosa and its downregulation by endotoxin. *Life Sci* 1997;61:1323–1329.
 21. Liu SF, Adcock IM, Old RW, et al. Differential regulation of the constitutive and inducible nitric oxide synthase mRNA by lipopolysaccharide treatment in vivo in the rat. *Crit Care Med* 1996;24:1219–1225.
 22. Michel T, Feron O. Nitric oxide synthases: which, where, how, and why? *J Clin Invest* 1997;100:2146–2152.
 23. Michel JB, Feron O, Sase K, et al. Caveolin versus calmodulin. Counterbalancing allosteric modulators of endothelial nitric oxide synthase. *J Biol Chem* 1997;272:25907–25912.
 24. Shah V, Toruner M, Haddad F, et al. Impaired endothelial nitric oxide synthase activity associated with enhanced caveolin binding in experimental cirrhosis in the rat. *Gastroenterology* 1999;117:1222–1228.
 25. Feron O, Michel JB, Sase K, et al. Dynamic regulation of endothelial nitric oxide synthase: complementary roles of dual acylation and caveolin interactions. *Biochemistry* 1998;37:193–200.
 26. Coccheri S, Nazzari M. Defibrotide as a possible anti-ischemic drug. *Semin Thromb Hemost* 1996;22(suppl 1):9–14.
 27. Ayuse T, Brienza N, Revelly JB, et al. Role of nitric oxide in porcine liver circulation under normal and endotoxemic conditions. *J Appl Physiol* 1995;78:1319–1329.
 28. Mathie RT, Ralevic V, Alexander B, et al. Nitric oxide is the mediator of ATP-induced dilatation of the rabbit hepatic arterial vascular bed. *Br J Pharmacol* 1991;103:1602–1606.
 29. Pannen BH, Bauer M. Differential regulation of hepatic arterial and portal venous vascular resistance by nitric oxide and carbon monoxide in rats. *Life Sci* 1998;62:2025–2033.
 30. Mittal MK, Gupta TK, Lee FY, et al. Nitric oxide modulates hepatic vascular tone in normal rat liver. *Am J Physiol* 1994;267:G416–G422.
 31. Zhang JX, Pegoli WJ, Clemens MG. Endothelin-1 induces direct constriction of hepatic sinusoids. *Am J Physiol* 1994;266:G624–G632.
 32. Shibayama Y, Nakata K. Role of septal fibrosis in development of hepatic circulatory disturbance in the presence of liver cell enlargement. *Liver* 1992;12:84–89.
 33. Harbrecht BG, Billiar TR, Stadler J, et al. Inhibition of nitric oxide synthesis during endotoxemia promotes intrahepatic thrombosis and an oxygen radical-mediated hepatic injury. *J Leukoc Biol* 1992;52:390–394.
 34. Harbrecht BG, Wu B, Watkins SC, et al. Inhibition of nitric oxide synthase during hemorrhagic shock increases hepatic injury. *Shock* 1995;4:332–337.
 35. Saetre T, Gundersen Y, Thiernemann C, et al. Aminoethylisothiourea, a selective inhibitor of inducible nitric oxide synthase activity, improves liver circulation and oxygen metabolism in a porcine model of endotoxemia. *Shock* 1998;9:109–115.
 36. Thiernemann C, Ruetten H, Wu CC, et al. The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br J Pharmacol* 1995;116:2845–2851.
 37. Wray GM, Millar CG, Hinds CJ, et al. Selective inhibition of the activity of inducible nitric oxide synthase prevents the circulatory failure, but not the organ injury/dysfunction, caused by endotoxin. *Shock* 1998;9:329–335.
 38. Ou J, Carlos TM, Watkins SC, et al. Differential effects of non-selective nitric oxide synthase (NOS) and selective inducible NOS inhibition on hepatic necrosis, apoptosis, ICAM-1 expression, and neutrophil accumulation during endotoxemia. *Nitric Oxide* 1997;1:404–416.
 - 38a. Ghosh S, Gachhui C, Crooks C, et al. Interaction between caveolin-1 and reductase domain of endothelial nitric-oxide synthase. Consequences for catalysis. *J Biol Chem* 1998;273:22267–22271.
 39. Yu Q, Shao R, Qian HS, et al. Gene transfer of the neuronal NO synthase isoform to cirrhotic rat liver ameliorates portal hypertension. *J Clin Invest* 2000;105:741–748.
 40. Rockey DC, Chung JJ. Inducible nitric oxide synthase in rat hepatic lipocytes and the effect of nitric oxide on lipocyte contractility. *J Clin Invest* 1995;95:1199–1206.
 41. Macedo MP, Lauth WW. Shear-induced modulation of vasoconstriction in the hepatic artery and portal vein by nitric oxide. *Am J Physiol* 1998;274:G253–G260.

42. Lee JW, Filkins JP. Exogenous ATP and hepatic hemodynamics in the perfused rat liver. *Circ Shock* 1988;24:99–110.
43. Higuchi H, Satoh T. Endothelin-1 induces vasoconstriction and nitric oxide release via endothelin ET(B) receptors in isolated perfused rat liver. *Eur J Pharmacol* 1997;328:175–182.
44. Gandhi CR, Sproat LA, Subbotin VM. Increased hepatic endothelin-1 levels and endothelin receptor density in cirrhotic rats. *Life Sci* 1996;58:55–62.
45. Sonin NV, Garcia-Pagan JC, Nakanishi K, et al. Patterns of vasoregulatory gene expression in the liver response to ischemia/reperfusion and endotoxemia. *Shock* 1999;11:175–179.
46. Clemens MG, Bauer M, Pannen BH, et al. Remodeling of hepatic microvascular responsiveness after ischemia/reperfusion. *Shock* 1997;8:80–85.
47. Bauer M, Bauer I, Sonin NV, et al. Functional significance of endothelin B receptors in mediating sinusoidal and extrasinusoidal effects of endothelins in the intact rat liver [see comments]. *Hepatology* 2000;31:937–947.
48. Vallance P, Moncada S. Role of endogenous nitric oxide in septic shock. *New Horiz* 1993;1:77–86.
49. Saarela J, Rehn M, Oikarinen A, et al. The short and long forms of type XVIII collagen show clear tissue specificities in their expression and location in basement membrane zones in humans. *Am J Pathol* 1998;153:611–626.
50. Liu P, McGuire GM, Fisher MA, et al. Activation of Kupffer cells and neutrophils for reactive oxygen formation is responsible for endotoxin-enhanced liver injury after hepatic ischemia. *Shock* 1995;3:56–62.
51. Fukatsu K, Saito H, Han I, et al. Nitric oxide donor decreases neutrophil adhesion in both lung and peritoneum during peritonitis. *J Surg Res* 1998;74:119–124.
52. Liu P, Yin K, Nagele R, et al. Inhibition of nitric oxide synthase attenuates peroxynitrite generation, but augments neutrophil accumulation in hepatic ischemia-reperfusion in rats. *J Pharmacol Exp Ther* 1998;284:1139–1146.
53. Liu P, Xu B, Hock CE, et al. NO modulates P-selectin and ICAM-1 mRNA expression and hemodynamic alterations in hepatic I/R. *Am J Physiol* 1998;275:H2191–H2198.
54. Farhood A, McGuire GM, Manning AM, et al. Intercellular adhesion molecule 1 (ICAM-1) expression and its role in neutrophil-induced ischemia-reperfusion injury in rat liver. *J Leukoc Biol* 1995;57:368–374.
55. Jaeschke H, Smith CW, Clemens MG, et al. Mechanisms of inflammatory liver injury: adhesion molecules and cytotoxicity of neutrophils. *Toxicol Appl Pharmacol* 1996;139:213–226.
56. Suematsu M, Kashiwagi S, Sano T, et al. Carbon monoxide as an endogenous modulator of hepatic vascular perfusion. *Biochem Biophys Res Commun* 1994;205:1333–1337.
57. Suematsu M, Goda N, Sano T, et al. Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. *J Clin Invest* 1995;96:2431–2437.
58. Bauer M, Pannen BHJ, Bauer I, et al. Evidence for a functional link between stress response and vascular control in hepatic portal circulation. *Am J Physiol* 1996;271:G929–G935.
59. Downard PJ, Wilson MA, Spain DA, et al. Heme oxygenase-dependent carbon monoxide production is a hepatic adaptive response to sepsis. *J Surg Res* 1997;71:7–12.
60. Vachharajani TJ, Work J, Issekutz AC, et al. Heme oxygenase modulates selectin expression in different regional vascular beds. *Am J Physiol Heart Circ Physiol* 2000;278:H1613–H1617.
61. Suematsu M, Ishimura Y. The heme oxygenase-carbon monoxide system: a regulator of hepatobiliary function. *Hepatology* 2000;31:3–6.
62. West MA, Keller GA, Hyland BJ, et al. Hepatocyte function in sepsis: Kupffer cells mediate a biphasic protein synthesis response in hepatocytes after exposure to endotoxin or killed *Escherichia coli*. *Surgery* 1985;98:388–395.
63. Billiar TR, Curran RD, Ferrari FK, et al. Kupffer cell: hepatocyte cocultures release nitric oxide in response to bacterial endotoxin. *J Surg Res* 1990;48:349–353.
64. Chartrain NA, Geller DA, Koty PP, et al. Molecular cloning, structure, and chromosomal localization of the human inducible nitric oxide synthase gene. *J Biol Chem* 1994;269:6765–6772.
65. Ferrante A, Jenkin CR, Reade PC. Changes in the activity of the reticulo-endothelial system of rats during an infection with *T. lewisi*. *Aust J Exp Biol Med Sci* 1978;56:47–59.
66. Curran RD, Ferrari FK, Kispert PH, et al. Nitric oxide and nitric oxide-generating compounds inhibit hepatocyte protein synthesis. *FASEB J* 1991;5:2085–2092.
67. Thelen M, Schulz D, Schild H, et al. [Changes in liver haemodynamics after mesenterico-caval dacron prosthesis anastomosis (“H-shunt”) in portal hypertension (author’s transl)]. *Anderungen der Leberhamodynamik nach mesenterikokavaler Dacron-Prothesen-Anastomose (sog. “H-Shunt”) bei portaler Hypertension*. *ROFO* 1978;128:423–431.
68. Ou J, Molina L, Kim YM, et al. Excessive NO production does not account for the inhibition of hepatic gluconeogenesis in endotoxemia. *Am J Physiol* 1996;271:G621–G628.
69. Mahnke PF, Keitel R, Otto U. [Morphological studies on swine livers after extracorporeal perfusion]. *Morphologische Untersuchungen an Schweinelebern nach extrakorporaler Perfusion*. *Z Exp Chir* 1978;11:95–102.
70. Szabo C. Potential role of the peroxynitrate-poly(ADP-ribose) synthetase pathway in a rat model of severe hemorrhagic shock. *Shock* 1998;9:341–344.
71. Wang K, Deutschman CS, Clemens MG, et al. Reciprocal expression of phosphoenolpyruvate carboxykinase and acute phase genes during acute inflammation. *Shock* 1995;3:204–209.
72. Fraser R, Bosanquet AG, Day WA. Filtration of chylomicrons by the liver may influence cholesterol metabolism and atherosclerosis. *Atherosclerosis* 1978;29:113–123.
73. Fisch C, Robin MA, Letteron P, et al. Cell-generated nitric oxide inactivates rat hepatocyte mitochondria in vitro but reacts with hemoglobin in vivo. *Gastroenterology* 1996;110:210–220.
74. Giulivi C. Functional implications of nitric oxide produced by mitochondria in mitochondrial metabolism. *Biochem J* 1998;332:673–679.
75. Kantrow SP, Taylor DE, Carraway MS, et al. Oxidative metabolism in rat hepatocytes and mitochondria during sepsis. *Arch Biochem Biophys* 1997;345:278–288.
76. Kurose I, Kato S, Ishii H, et al. Nitric oxide mediates lipopolysaccharide-induced alteration of mitochondrial function in cultured hepatocytes and isolated perfused liver. *Hepatology* 1993;18:380–388.
77. Balakirev MY, Khramtsov VV, Zimmer G. Modulation of the mitochondrial permeability transition by nitric oxide. *Eur J Biochem* 1997;246:710–718.
78. Hortelano S, Dallaporta B, Zamzami N, et al. Nitric oxide induces apoptosis via triggering mitochondrial permeability transition. *FEBS Lett* 1997;410:373–377.
- 78a. Balakirev MY, Khramtsov VV, Zimmer G. Modulation of the mitochondrial permeability transition by nitric oxide. *Eur J Biochem* 1997;246:710–718.
79. Szabo C, Ohshima H. DNA damage induced by peroxynitrite: subsequent biological effects. *Nitric Oxide* 1997;1:373–385.
80. Szabo C. DNA strand breakage and activation of poly-ADP ribosyltransferase: a cytotoxic pathway triggered by peroxynitrite. *Free Radic Biol Med* 1996;21:855–869.
81. Costantini P, Petronilli V, Colonna R, et al. On the effects of paraquat on isolated mitochondria. Evidence that paraquat

- causes opening of the cyclosporin A-sensitive permeability transition pore synergistically with nitric oxide. *Toxicology* 1995;99:77–88.
82. Packer MA, Murphy MP. Peroxynitrite causes calcium efflux from mitochondria which is prevented by cyclosporin A. *FEBS Lett* 1994;345:237–240.
 83. Kim YM, Bombeck CA, Billiar TR. Nitric oxide as a bifunctional regulator of apoptosis. *Circ Res* 1999;84:253–256.
 84. Kim YM, Talanian RV, Billiar TR. Nitric oxide inhibits apoptosis by preventing increases in caspase-3-like activity via two distinct mechanisms. *J Biol Chem* 1997;272:31138–31148.
 85. Duca C, Duca S, Uray Z, et al. Improvement of perfusion flow in the isolated rat liver under the influence of streptase. Auto-historadiographic aspects of 125I-labelled fibrinogen deposition. *Arzneimittelforschung* 1978;28:407–409.
 86. Kim YM, de Vera ME, Watkins SC, et al. Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-alpha-induced apoptosis by inducing heat shock protein 70 expression. *J Biol Chem* 1997;272:1402–1411.
 87. Li J, Billiar TR, Talanian RV, et al. Nitric oxide reversibly inhibits seven members of the caspase family via S-nitrosylation. *Biochem Biophys Res Commun* 1997;240:419–424.
 88. Ambs S, Ogunfusika MO, Merriam WG, et al. Up-regulation of inducible nitric oxide synthase expression in cancer-prone p53 knockout mice. *Proc Natl Acad Sci USA* 1998;95:8823–8828.
 89. Storch W. [About the differentiation of antibodies against the connective tissue (author's transl)]. Zur Differenzierung von Antikörpern gegen Bindegewebe. *Acta Histochem* 1978;62:57–67.
 90. Rai RM, Lee FY, Rosen A, et al. Impaired liver regeneration in inducible nitric oxide synthase-deficient mice. *Proc Natl Acad Sci USA* 1998;95:13829–13834.
 91. Bartsch H, Ohshima H, Pignatelli B, et al. Endogenously formed N-nitroso compounds and nitrosating agents in human cancer etiology. *Pharmacogenetics* 1992;2:272–277.
 92. Kew MC, Minick OT, Bahu RM, et al. Ultrastructural changes in the liver in heatstroke. *Am J Pathol* 1978;90:609–618.
 93. Ohshima H, Bartsch H. Chronic infections and inflammatory processes as cancer risk factors: possible role of nitric oxide in carcinogenesis. *Mutat Res* 1994;305:253–264.