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Review article

Biomechanisms of cocaine-induced hepatocyte injury mediated by the formation of reactive metabolites*

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Abstract. Cocaine is an intrinsic hepatotoxin in laboratory animals, and there is growing evidence that high doses of cocaine can precipitate hepatic necrosis in humans. The rodent model of cocaine hepatotoxicity is commensurate with the concept that a multistep, mainly cytochrome P-450 dependent *N*-oxidative pathway is responsible for the expression of hepatocellular injury. Among the possible biomechanisms by which cocaine exerts its cytotoxic effects, direct oxidative damage by reactive oxygen species generated by redox cycling during the metabolic cascade seems most important. The role of the ensuing lipid peroxidation and protein thiol oxidatioin is less clear. Similarly, the functional role of irreversible (covalent) binding of a not yet defined electrophilic cocaine intermediate to hepatocellular proteins remains enigmatic so long as the critical molecular targets have not been identified. Finally, glutathione plays a pivotal protective role against cocaine-induced hepatic injury. Interactions with ethanol or inducers of the expression of the cytochrome P-450IIB subfamily can potentiate cocaine hepatotoxicity. Thus, the net amount of the ultimate reactive species seems to determine the severity of the hepatic lesions and to be responsible for the marked interspecies, interstrain, and sex differences. Recent advances in culture techniques of hepatocytes and precision-cut liver slices from various species including man have made it possible to correlate cocaine biotransformation with cytotoxicity and to selectively study the putative cellular mechanisms. Clearly, more studies are necessary to further illuminate our understanding of the role of the biochemical and molecular events precipitating hepatic necrosis during cocaine-mediated hepatotoxicity.

Key words: Cocaine – Mechanisms of hepatotoxicity – Cytochrome P-450IIB – Irreversible binding – Reactive oxygen species

* Dedicated to Professor Dr. Gerhard Zbinden on the occasion of his retirement

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Introduction

The increasing use of cocaine as a habitual or recreational drug has reached dramatic dimensions and is associated with socioeconomical, neuropsychiatric and medical consequences, although it is difficult to assess exact rates of prevalence (Abelson and Miller 1985). In parallel with the increasing cocaine use has been a sharp increase in the number of cocaine-related hospital admissions and deaths. This is predominantly due to cardiovascular, central nervous and neuromuscular system toxicity but also includes complications of renal and pulmonary injury, as well as possible teratogenic effects (reviewed by Farrar and Kearns 1989; Johanson and Fischman 1989; Loper 1989; Fleming et al. 1990; Rossi 1990). However, there is increasing clinical evidence that, besides these major sites, the liver is also a target organ of cocaine-related toxicity. Although a recent report indicates that cocaine use is rarely associated with significant liver function test abnormalities (Tabasco-Minguillan et al. 1990), several other studies document that high doses of cocaine can precipitate massive hepatic necrosis as reflected by profound increases in serum alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT) and bilirubin levels, and by perivenous, midzonal or periportal parenchymal necrosis (Marks and Chapple 1967; Perino et al. 1987; Kanel et al. 1990; Kokko 1990; Wanless et al. 1990). Collectively, these recent studies indicate that cocaine is potentially hepatotoxic in humans.

The clinical and histopathological pattern of cocaine-related liver toxicity in humans is similar to earlier studies performed in experimental animals (Shuster et al. 1977; Evans and Harbison 1978; Thompson et al. 1979; Freeman and Harbison 1981a; Kloss et al. 1982b; Evans 1983; Kloss et al. 1984; Thompson et al. 1984). For example, liver injury in mice following an acute dose of cocaine was manifested by strikingly increased serum transaminase levels and both microvesicular fatty degeneration of hepatocytes and parenchymal necrosis. The extent of the toxic insult and the variance in zonal location was largely depen-

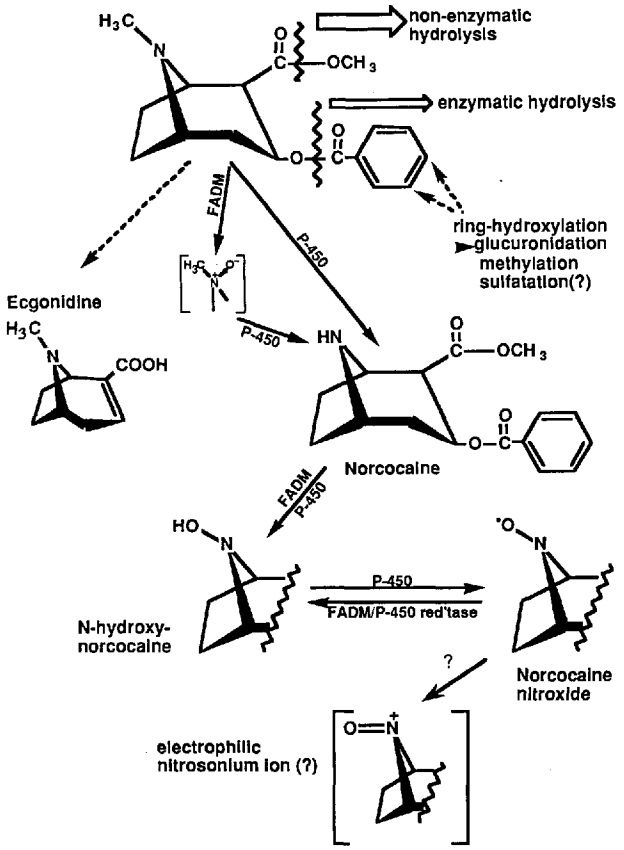

Fig. 1. Metabolic pathways of cocaine hydrolytic inactivation to non-toxic metabolites and *N*-oxidative activation to reactive intermediates. *P-450*, cytochrome P-450; *FADM*, flavine-containing monooxygenases; *P-450 red'tase*, NADPH cytochrome P-450 reductase

dent on the presence of various types of drug biotransformation inducers.

Due to the progress made in culture techniques of both isolated hepatocytes and precision-cut liver slices from various species, cocaine was the subject of a number of in vitro studies relating to metabolism (Bouis and Boelsterli 1990; Roberts et al. 1990), elucidation of mechanisms of toxicity (Donnelly et al. 1988; Göldlin and Boelsterli 1991a, b) or species-specific responses to the toxic insult (Connors et al. 1990).

Several lines of evidence currently suggest that reactive cocaine metabolites are responsible for the cytotoxic effects caused by cocaine. The biomechanisms by which these intermediates exert their toxic effects remains less clear. Two basic mechanisms have been evoked to account for the cytotoxic effects of cocaine. First, promotion of lipid peroxidation was suggested to mediate cocaine-induced hepatotoxicity by damaging cellular membranes through compromising key hepatic enzyme activities (Kloss et al. 1984). Second, liver damage has been related to covalent interaction of a chemically reactive cocaine intermediate with critical tissue proteins (Evans 1983; Bouis and Boelsterli 1990). However, the relative contribution of these hypothetical mechanisms, and the sequence of early events during cocaine-induced hepatotoxicity remains unclear. Furthermore, the location and molecular nature of this interaction with cellular targets remains enigmatic. Thus, it is not clear whether these observed biochemical changes are causally related to cocaine-induced killing of hepatocytes or whether they only represent epiphenomena.

This article summarizes the present information on the bioactivation of cocaine to reactive intermediates and the significance of these in precipitating toxic liver injury, and critically reviews possible cellular or molecular biomechanisms of toxicity.

Metabolism and bioactivation of cocaine

When cocaine hepatotoxicity began to emerge as a subject of interest in the early 1970s, it was already known that cocaine was extensively metabolized. Benzoyl ecgonine and ecgonine, two hydrolytic products, had been identified as cocaine metabolites in tissues or urine of animals and humans. Furthermore, $^{14}CO_2$ release from (N-$^{14}CH_3$)-cocaine-treated mice indicated the formation of norcocaine and norecgonine by oxidative *N*-demethylation (Werner 1961).

Metabolic pathways

Cocaine is metabolized via three basic pathways. The hydrolytic pathway of cocaine degradation is quantitatively most important (Fig. 1). Benzoyl ecgonine, formed non-enzymatically, and ecgonine methylester, generated by serum cholinesterase and hepatic esterases (Stewart et al. 1979), are by far the most abundant urinary metabolites in man, representing 30–45% and 15–35%, respectively, of the total dose administered (Inaba et al. 1978; Ambre et al. 1988). These and other identified hydrolytic metabolites were found to lack hepatotoxic potential (Thompson et al. 1979; Freeman and Harbison 1981b).

Second, the quantitatively less important *N*-oxidative pathway (Fig. 1) was recognized as the bioactivating cascade leading to cocaine hepatotoxicity. Evidence stemmed from studies demonstrating enhancement of the hepatotoxic effects in mice by inducing mixed function oxygenases (Evans and Harbison 1978; Thompson et al. 1979) or by metabolic channeling towards oxidative metabolism by means of an esterase inhibitor (Thompson et al. 1979). In contrast, inhibition of mixed function oxygenases attenuated cocaine cytotoxicity (Thompson et al. 1979; Evans 1983). Cocaine is *N*-demethylated to norcocaine and further converted to *N*-hydroxynorcocaine. The presence of norcocaine has been demonstrated in man (Inaba et al. 1978; Jindal et al. 1978; Lowry et al. 1979), whereas *N*-hydroxynorcocaine has been identified in rats and mice only (Evans 1983; Shuster et al. 1983; Jindal and Lutz 1986; Benuck et al. 1988). When administered to mice at equimolar doses, norcocaine was demonstrated to be more hepatotoxic than the cocaine parent molecule, and *N*-hydroxynorcocaine was the most potent species of all (Thompson et al. 1979). The final metabolite in the cascade is the norcocaine nitroxide radical, which is formed by one-electron oxidation from *N*-hydroxynorcocaine and which has been detected by ESR techniques in hepatic

microsomal suspensions from mice (Evans and Johnson 1981), rats and hamsters (Rauckman et al. 1982b). Although norcocaine nitroxide is often referred to as the ultimate reactive species that could covalently bind to hepatic proteins or initiate lipid peroxidation (Thompson et al. 1979), Rauckman et al. (1982a) showed that synthesized norcocaine nitroxide was poorly reactive and neither formed adducts with proteins or glutathione. Norcocaine nitroxide can be reduced again to *N*-hydroxynorcocaine, and it has therefore been suggested that *N*-hydroxynorcocaine and norcocaine nitroxide undergo a futile redox cycle that generates superoxide anion radicals and H_2O_2, thus causing hepatotoxicity via lipid peroxidation rather than by a reactive metabolite per se (Rauckman and Rosen 1982; Rauckman et al. 1982a; Kloss et al. 1984). Nevertheless, the presence of irreversibly bound cocaine adducts to hepatic macromolecules is commensurate with a highly electrophilic intermediate. In the search for the ultimate metabolite, Charkoudian and Shuster (1985) proposed the norcocaine nitrosonium ion (oxammonium ion) as the ultimate binding species. Based on electrochemical studies in vitro, the authors suggested that the nitrosonium ion could be formed by oxidation of norcocaine nitroxide. Once formed, the electrophilic intermediate would immediately react with water to give *N*-hydroxynorcocaine or interact with other cell constituents to form protein adducts or lipid peroxidation.

Finally, arylhydroxylation followed by methylation (Smith 1984a, b; Smith et al. 1984), glucuroconjugation and possibly sulfate conjugation (Jindal and Lutz 1986; Zhang and Foltz 1990) of the phenolic metabolites was recognized as a third pathway in cocaine metabolism in rats and in humans. In the rat, phenolic compounds and tropane-hydroxylated metabolites have been tentatively identified (Nayak et al. 1976). Conjugation reactions of toxicological interest, such as *N*-sulfate conjugation or conjugation with glutathione, have not been reported.

Furthermore, analysis by GC-MS of human urine and bile revealed ecgonidine methylester (Lowry et al. 1979) and ecgonidine (Zhang and Foltz 1990) as additional metabolites of cocaine. The formation of these compounds is poorly understood.

Role of cytochrome P-450 and FAD-containing monooxygenases

The multistep *N*-oxidative pathway is catalyzed by both cytochrome P-450 and FAD-containing monooxygenases (Fig. 1). P-450-dependent monooxygenases are involved in all three metabolic steps up to norcocaine nitroxide formation (Shuster et al. 1983). An emerging area of research is the implication of individual forms of P-450 in cocaine bioactivation and cytotoxicity. Several lines of evidence suggest that the phenobarbital-inducible P-450IIB subfamily is involved in microsomal cocaine *N*-demethylation, with a rather high apparent K_m of approximately 1 mM (Boelsterli et al. 1990, 1991). This was inferred from inhibition experiments using isozyme-selective substrates and isozyme-specific polyclonal antibodies. FAD-containing monooxygenases are involved both in the first (Kloss et al. 1983a) and in the second step of oxidative metabolism (Kloss et al. 1982a). Norcocaine is an excellent substrate (apparent K_m 15 μM) in mouse hepatic microsomes (Kloss et al. 1982a), whereas cocaine is a poor substrate for FAD-containing monooxygenases; the apparent K_m in a purified guinea pig enzyme system was 2.8 mM (Yuno et al. 1990).

Factors modulating cocaine metabolism and hepatotoxicity

Several intracellular and extracellular biochemical variables could theoretically modulate cocaine biotransformation and hepatotoxicity, and some of them have been experimentally confirmed to be linked to hepatocellular viability. The ratio of the cocaine-inactivating (hydrolysis by esterases) to the cocaine-activating metabolic pathways, the cellular antioxidant status, the cellular non-protein thiol and protein thiol status, and interaction with other toxic drugs or chemicals all may modulate cocaine metabolism and hepatotoxicity. Aside from serum cholinesterase activity, factors affecting the genetically determined and/or factors influencing regulative expression of those monooxygenases involved in the *N*-oxidative pathway are of prime importance.

Rate of metabolite formation: dependence on species, strain, sex, developmental stage

There is a striking species difference in the hepatotoxic response to cocaine. The species most susceptible and, therefore, widely used as an experimental model, is the mouse (Evans and Harbison 1978; Thompson et al. 1979). Acute doses of 60 mg/kg cocaine, administered intraperitoneally, cause severe hepatic injury. In contrast, cocaine administration failed to induce hepatic injury in Sprague-Dawley rats (Freeman and Harbison 1981a; Evans 1983). This species difference was corroborated in isolated hepatocytes from mice and rats. Cocaine concentrations of 500 μM caused an almost complete loss of viability in mouse hepatocytes, whereas rat hepatocytes remained unaffected several hours (Donnelly et al. 1988). Human hepatocytes seem to be more resistant towards cocaine than mice or rat hepatocytes, as demonstrated by higher K^+ retention and minimal Ca^{++} uptake in cultured precision-cut liver slices (Connors et al. 1990). Large variations in the expression of cocaine-induced hepatotoxicity were not only found between different species but also among different strains. For example, several strains of mice (Thompson et al. 1984; Boyer et al. 1988; Connors et al. 1990; Smolen and Smolen 1990) and rats (Watanabe et al. 1987) exhibited significant differences in the levels of plasma ALAT or ASAT activities, indicative of hepatic injury due to acute, chronic, or subchronic cocaine administration. Furthermore, male mice or rats are more prone to develop cocaine-induced liver damage than females (Thompson et al. 1984; Watanabe et al. 1987; Boyer et al. 1988; Smolen and Smolen 1990). In addition, the expression of cocaine hepatotoxicity has been demonstrated to be

dependent on the ontogenetic stage of development. Toxicity appeared with the onset of the adolescent phase (30 days of age) and persisted in adult male mice, whereas liver injury was low or absent in immature mice of both sexes or in adult female mice (Smolen and Smolen 1990).

The underlying biochemical mechanisms for these large interspecies, interstrain, sex and age-dependent variations in cocaine hepatotoxicity are largely unknown yet important factors for the prediction of hepatotoxicity and for conducting a risk assessment in humans. It can be inferred from the current evidence that both the rate of hydrolytic inactivation and the rate of cocaine bioactivation are major determinants for the observed differences in sensitivity. Several experimental findings support this concept. First, plasma esterase activity has been shown to vary among different species and to be inversely correlated with toxicity. For example, the percentage of remaining cocaine and norcocaine incubated for 1 h with plasma samples from various species was highest in the rat, followed by the dog, and was lowest in human plasma (Bouis et al. 1990). Second, the rate of cocaine oxidative biotransformation in microsomal preparations has been shown to correlate with the extent of serum transaminase levels following in vivo administration of cocaine. For example, cocaine *N*-demethylase activity was found to be greater in spontaneously hypertensive rats than in Wistar Kyoto rats (Watanabe et al. 1987). In addition, rates of cocaine *N*-demethylase in vivo are highest in the mouse (45% of the total dose administered; Werner 1961), intermediate in the rat (17%; Englert et al. 1976), and lowest in man (2.4–6.2%; Inaba et al. 1978). However, in contrast to these findings is a recent report (Leibman et al. 1990) providing evidence that, at least among two different mice strains, the interstrain and sex-dependent variations in the hepatotoxic response to cocaine cannot be explained by differential activities of cocaine metabolizing enzymes. Specifically, the activities of both cocaine-*N*-demethylase and FAD-containing monooxygenase exhibited no consistent positive correlation with sex-or strain-dependent differences in susceptibility to the toxic insult. Thus, the regulatory role of basic rates of these *N*-oxidizing enzyme activities in mediating hepatic toxicity needs further clarification. These basic rates of drug biotransformation can, however, be dramatically changed by induction of the functional expression of the P-450 enzymes involved in cocaine bioactivation.

Rate of metabolite formation: induction or inhibition of P-450

A quantitatively minor pathway in cocaine biotransformation is the *N*-oxidative metabolism mediated by cytochrome P-450 and FAD-containing monooxygenases. When these microsomal drug-metabolizing enzymes are induced, or when the metabolism is channeled by inhibition of the major hydrolytic pathways, marked increases in the rates of oxidative biotransformation were observed that were paralleled by exacerbation of cocaine hepatotoxicity. For example, phenobarbital pretreatment in mice increased plasma levels of ALAT or ASAT following cocaine administration (Thompson et al. 1979; Evans 1983). Specifically, the toxic effects were manifested as delayed hepatotoxicity, whereas the acute toxicity (3 h lethality due to CNS toxicity) was reduced (Evans and Harbison 1978). Similarly, phenobarbital pretreatment enhanced the cytotoxic effects in cultured rat hepatocytes (Roberts et al. 1990; Göldlin and Boelsterli 1991 a, b) or in cultured liver slices from rats or mice (Connors et al. 1990). That rat hepatocytes in culture were still able to express metabolic changes induced in vivo was demonstrated by markedly increased norcocaine formation in cells from Aroclor 1254-pretreated rats (Bouis and Boelsterli 1990).

Conversely, cocaine-associated hepatotoxicity is attenuated both in vivo and in vitro when the cytochrome P-450 dependent monooxygenases involved in cocaine metabolism are inhibited. For example, administration of SKF-525A reduced plasma ASAT levels in cocaine-treated mice (Thompson et al. 1979) or attenuated cocaine-mediated liver necrosis in phenobarbital-pretreated mice (Evans 1983). Similarly, chloramphenicol or iproniazid (Thompson et al. 1979), metyrapone (Kloss et al. 1982b), or cimetidine (Peterson et al. 1983) administration in mice protected against cocaine-induced increases in plasma transaminase levels. In cultured hepatocytes, addition of SKF-525A protected the cells against the cytotoxic effects of cocaine as evidenced by great reductions in LDH release (Roberts et al. 1990) or by restoration of cocaine or norcocaine-induced impairment of protein biosynthesis (Bouis and Boelsterli 1990).

Other types of inducing agents that can lead to potentiation of liver toxicity include ethanol (Lieber 1990). Evaluation of the interaction of cocaine with ethanol, however, is suggestive of the presence of a complex pattern of cellular changes other than induction of the ethanol-inducible P-450 forms alone.

Ethanol interaction

Several recent reports have identified alcohol in combination with cocaine as one of the most common drug use patterns in the general US population (Kreek and Stimmel 1984; Grant and Harford 1990). Concurrent use of both substances may not only place these subgroups at higher risk for neuropsychological impairment and exacerbation of morbidity but also may play a role in metabolic interactions. Since alcohol is a well known inducer of P-450, it can be surmised that chronic alcohol consumption may induce the rate of cocaine metabolism, thereby augmenting reactive metabolite formation associated with acute liver injury. However, the role of ethanol as a possible inducer of those P-450 forms that are involved in cocaine bioactivation in man has not been determined.

In contrast, experimental models have clearly demonstrated a promoting effect of ethanol on cocaine-mediated hepatotoxicity. For example, pretreatment of mice with an ethanol-containing liquid diet sensitized the animals to cocaine-induced hepatotoxicity, apparent as a latent (1–7 days) appearance of profound centrilobular necrosis and highly increased levels of serum ALAT (Smith et al. 1981). None of the pair-fed controls exhibited hepatic necrosis after cocaine doses of up to 60 mg/kg. In contrast,

the acute (3 h) lethality, due to central toxicity, was markedly reduced by the ethanol pretreatment. These findings are consistent with an increased rate of cocaine biotransformation.

It has tentatively been proposed that cocaine might be a substrate for the ethanol-inducible P-450IIE1 (Coon and Koop 1987; Boyer and Petersen 1990), but recent evidence seems to dispute this contention (Boelsterli et al. 1990, 1991). Although the rates of both microsomal cocaine *N*-demethylation and *p*-nitrophenol hydroxylation, which is a probe for the catalytic activity of P-450IIE1, were increased in ethanol-fed rats as compared to concurrent controls, both functionally active polyclonal anti-rat P-450IIE1 antibodies and substrates for P-450IIE1 failed to significantly inhibit cocaine metabolism and irreversible binding to microsomal proteins. In addition, pretreatment of rats with pyrazole, another inducer of P-450IIE1, did not enhance cocaine metabolism although this drug potentiated cocaine cytotoxicity in vitro (Boelsterli et al. 1991). Thus, it is unlikely that P-450IIE1 is involved in cocaine bioactivation. The enhancing effects of ethanol on cocaine-induced hepatotoxicity could rather be explained by induction of other P-450 forms (Johansson et al. 1988) or by a promoting effect of the induced P-450IIE1 on the formation of reactive oxygen species even in the absence of a substrate (Ekström and Ingelman-Sundberg 1989).

In view of a recent mouse study where not only chronic but also acute (3 g/kg, i.p.) ethanol treatment markedly increased serum ALAT activities (Boyer and Petersen 1990), it is likely that effects other than induction of P-450 could account for the promoting effects of ethanol on cocaine-mediated hepatotoxicity. For example, it is well known that ethanol reduces the hepatocellular glutathione (GSH) content (Fernandez-Checa et al. 1987). Furthermore, another effect linked to ethanol is the promotion of lipid peroxidation in the liver. Recently, evidence has been presented that acutely administered ethanol might increase superoxide anion radical production via xanthine oxidase activity and thus enhance cocaine hepatotoxicity by oxidative stress (Boyer et al. 1990). Finally, evidence has been presented that the presence of ethanol and cocaine in man leads to the formation of a new metabolite, "cocaethylene" (the ethyl homolog of cocaine), that was detected in urine (Rafla and Epstein 1979) and in human brain and liver tissue from cocaine-related sudden death cases (Hearn et al. 1990). However, no studies on the hepatotoxic potential of this metabolite have been reported.

Role of glutathione

Glutathione (GSH) has been implicated as a protective species in hepatocellular defense against reactive electrophilic intermediates from a variety of hepatotoxic drugs and chemicals. Glutathione acts both as a nucleophilic scavenger, converting electrophilic metabolites to thioether compounds, and as a substrate in the degradation of hydroperoxides (Reed 1990). A major protective role for GSH against cocaine-induced hepatic toxicity is suggested by a number of observations. First, the severity of cocaine-induced liver injury was potentiated following depletion of hepatic GSH with diethyl maleate or buthionine sulfoximine in vivo (Evans and Harbison 1978) or in vitro (Bouis and Boelsterli 1990; Göldlin and Boelsterli 1991 a, b). Second, administration of cysteine, the rate-limiting precursor for GSH synthesis, protected against cocaine-induced ASAT release in mice (Evans and Harbison 1978; Thompson et al. 1979). Third, administration of high doses of cocaine was correlated with moderately decreased levels of hepatic reduced thiol GSH (not lower than 40% of initial content) in some mice strains (Kloss et al. 1982b; Thompson et al. 1984; Suarez et al. 1986) and with increased concentrations of oxidized disulfide glutathione (GSSG) in isolated hepatocytes (Donnelly et al. 1988) suggesting both adduct formation with activated cocaine metabolites and partial oxidative GSH depletion. However, several experimental findings do not support the concept of a direct inverse correlation between GSH content and the degree of hepatotoxicity. For example, GSH depletion, brought about by acute cocaine administration, can occur independently of the expression of hepatic toxicity (Thompson et al. 1984; James et al. 1987). Furthermore, chronic administration of cocaine to mice can induce liver damage paralleled by increased rather than decreased levels of hepatic GSH which has been explained by compensatory de novo synthesis of GSH (Wiener and Reith 1990). Thus, the role of GSH as a crucial factor for the development of cocaine-induced hepatotoxicity is difficult to assess as long as GSH-cocaine adducts have not been found and as long as both cytosolic and mitochondrial non-protein (GSH) and protein thiol homeostasis has not been determined.

In addition, it has been demonstrated that part of the GSH depletion observed when cocaine is administered results from the release of epinephrine and norepinephrine from the adrenals, due to the sympathomimetic effects of cocaine (James et al. 1987). This could be responsible for the potentiating effect of epinephrine given to mice prior to cocaine (Shuster and Thompson 1985) and could be an important modulating factor in cocaine-induced hepatotoxicity.

Irreversible protein binding of cocaine metabolites

Although a large number of drugs and chemicals are known to be bioactivated to reactive metabolites that irreversibly bind to tissue macromolecules, for most of these compounds including cocaine, the relationship between irreversible protein binding and cytotoxicity remains still enigmatic. One of the reasons is that both the reactive species and the nucleophilic molecular targets are unknown. In contrast to recent studies with acetaminophen covalent protein binding (Birge et al. 1990), the selective target proteins that are attacked by electrophilic cocaine intermediates have not been determined. Similarly, the chemical nature of the reactive metabolites that attacks nucleophilic sites in proteins has not yet been clearly identified. Although it has been demonstrated that the ultimate reactive species was *N*-demethylated and retained its two ester groups (Evans 1983), attempts to attribute the binding properties to norcocaine nitroxide (Evans 1983) contrasted with other findings providing evidence that norcocaine nitroxide was unreactive in vitro both with dialyzed cytosol

and hepatic microsomes obtained from mice (Rauckman and Rosen 1982) and both with GSH or cysteine (Rauckman et al. 1982b). Alternatively, Charkoudian and Shuster (1985) suggested the nitrosonium ion of norcocaine, a highly electrophilic species, to play a major role in binding to subcellular constituents. It is unknown, however, whether such an intermediate is generated in vivo.

Irreversible binding of cocaine to hepatic protein was first described by Evans and Harbison (1978). Whereas the rate of irreversible protein binding could be increased several-fold by prior induction with phenobarbital in mice, rats or hamsters failed to exhibit increased amounts of irreversibly bound cocaine to liver macromolecules (Evans 1983). In mice, the extent of cocaine-protein adduct formation was paralleled by the degree of hepatic necrosis or by increases in serum ALAT activities, suggesting a direct causal relationship. However, depletion of hepatic GSH with diethylmaleate augmented the severity of cocaine-induced necrosis without affecting irreversible binding, which was a first indication that binding might reflect the rate of bioactivation rather than constituting a direct link to cytotoxicity. In accordance, administration of cysteine, a GSH precursor, reduced the severity of liver necrosis but had no effect on the binding of cocaine to hepatic protein.

In primary cultures of rat hepatocytes, Bouis and Boelsterli (1990) demonstrated that a high proportion of total cell-associated radiolabelled cocaine was irreversibly bound to cellular macromolecules. Binding was P-450 mediated and occurred in a temporal and concentration-dependent manner. Partial depletion of glutathione with buthionine sulfoximine increased the rate of protein binding. Again, irreversible protein binding of cocaine was associated with impairment of hepatocellular function, measured as a partial inhibition of protein biosynthesis. However, the causal significance of irreversible protein binding in cocaine-mediated hepatotoxicity still remains an area of controversy.

The nature of irreversible protein binding was further characterized in rat liver microsomes using tritiated cocaine and various inhibitors of P-450 monooxygenases (Boelsterli et al. 1990). Both ethanol and phenobarbital pretreatment increased the rate of irreversible binding, which is compatible with an induction of cocaine-metabolizing hepatic monooxygenases. To determine which P-450 forms were involved in cocaine bioactivation, isozyme-selective substrates of the P-450IIB and P-450IIE1 subfamilies, as well as isozyme-specific antibodies were used (Boelsterli et al. 1991). The rates of both cocaine *N*-demethylation and bioactivation, measured as irreversible binding, were mediated by P-450IIB but not by P-450IIE1.

Cocaine-induced generation of reactive oxygen species and lipid peroxidation

Lipid peroxidation has been implicated as an important biomechanism for the hepatotropic toxicity of a number of drugs and chemicals (Guengerich and Liebler 1985; Nelson and Pearson 1990). Specifically, the peroxidative decomposition of hepatocyte membrane phospholipids, due to oxidative stress, has been evoked to account for lethal cell injury (Farber et al. 1990). Ample evidence for the occurrence of lipid peroxidation during cocaine-mediated hepatotoxicity stems from in vivo data and from in vitro investigations in hepatocyte cultures and microsomal systems. However, the causative role of lipid peroxidation for the development of cocaine-induced hepatocyte killing remains controversial.

Supportive evidence for a major role of lipid peroxidation in cocaine-induced hepatic injury was provided by the findings that lipid peroxidation, assessed by both the formation of conjugated dienes and the generation of thiobarbituric acid-reactive substances (TBA-RS) in the liver, occurred prior to an increase in plasma aminotransferase activities in cocaine treated mice (Kloss et al. 1983b; Teaf et al. 1984). Furthermore, ultrastructural hepatocellular alterations in mice correlated with the previously observed increase in conjugated diene formation in microsomal lipids and resembled the morphologic changes seen in carbon tetrachloride-induced liver cell damage (Gottfried et al. 1986). Finally, the relative hepatotoxic potential of cocaine and its *N*-oxidized metabolites, norcocaine, *N*-hydroxynorcocaine, and norcocaine nitroxide, were shown to parallel the amount of TBA-RS generated during the incubation of microsomes with equimolar concentrations of the various metabolites (Rosen et al. 1982; Kloss et al. 1983b).

Based on a series of experiments, the stimulation of lipid peroxidation was demonstrated to be causally linked with cocaine metabolism (Rauckman and Rosen 1982; Rauckman et al. 1982a, b). Redox cycling between norcocaine nitroxide and *N*-hydroxynorcocaine was suggested to be related to the production of superoxide anion, at the expense of NADPH. In addition to the initiation of membrane lipid peroxidation by the formation of reactive oxygen species, NADPH depletion could decrease the cellular availability of reducing equivalents and thus impair the reduction of oxidized glutathione (GSSG) to reduced GSH. Thereby the deleterious effects of the reactive oxygen species would be further enhanced, since GSH is an important part of the cellular defense system against oxidative stress and lipid peroxidation (reviewed by Kloss et al. 1984).

While it can be inferred from these data that lipid peroxidation plays a causative role in the development of hepatic toxicity, a number of recent reports seem to dispute this assumption. For example, incubation of isolated hepatocytes with high concentrations of cocaine resulted in marked cytotoxicity, but the rises in TBA-RS were modest. Specifically, when 80% of the hepatocytes were killed, the TBA-RS values exceeded control values by only 20% and 60% in mice or rat hepatocytes, respectively. Furthermore, the addition of the antioxidant, *N,N'*-diphenylphenylene diamine (DPPD) decreased the extent of lipid peroxidation to below control levels but had no effect on the severity of cytotoxicity (Donnelly et al. 1988). Similarly, hepatic necrosis in mice did not need to elicit lipid peroxidation in the liver (Powell and Charles 1990) and could not be prevented by pretreating the animals with the antioxidant butylated hydroxytoluene (BHT), suggesting that lipid peroxidation may not be a critical determinant in cocaine-induced hepatotoxicity.

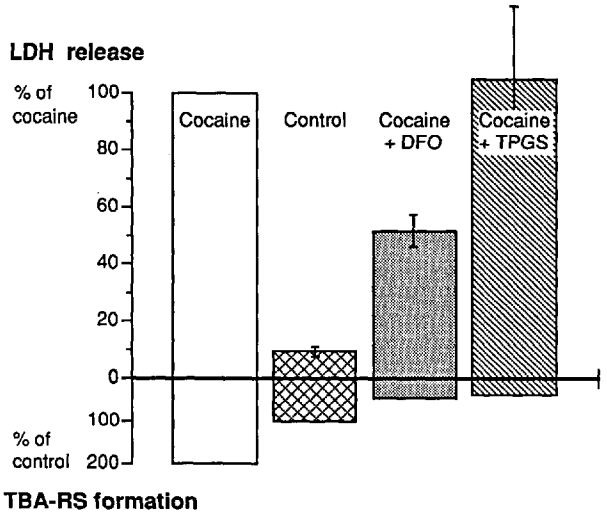

Fig. 2. Reactive oxygen species-induced cytotoxicity independent of lipid peroxidation. Primary short-term cultured rat hepatocytes derived from phenobarbital-pretreated rats were incubated in the presence or absence of cocaine (330 μM) for 24 h. To some of the culture dishes, the iron chelator deferoxamine (DFO) or the antioxidant α-tocopherol polyethyleneglycol succinate (TPGS) were added at 5 mM. LDH release is expressed as percent of extracellular LDH in cocaine-treated cultures, normalized to 100% (range: 22–62% of total LDH). The values are means ± SD of three experiments. Lipid peroxidation is expressed as percent of thiobarbituric acid-reactive substances (TBA-RS) in control cultures. The values are means of two experiments. Control (100%): 0.59 nmol TBA-RS/mg protein/24 h

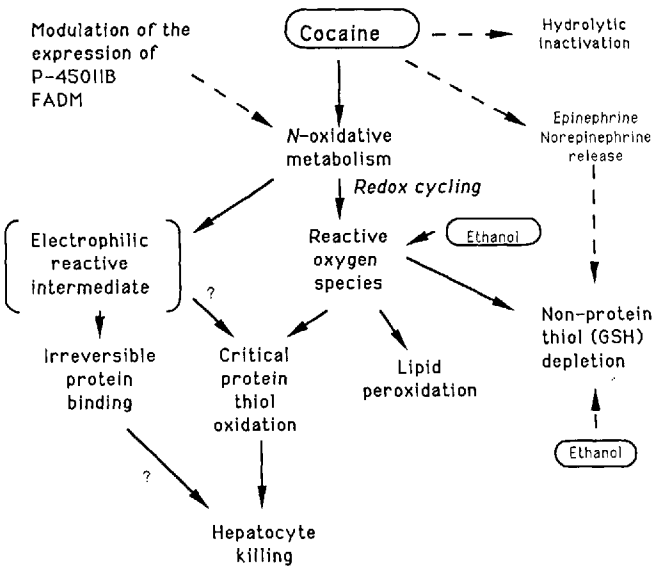

Fig. 3. Proposed biomechanistic pathways and some of the modulating factors in cocaine-mediated hepatocellular injury

To shed more light on the roles of peroxidative and non-peroxidative mechanisms for cocaine-mediated hepatic injury, the time-course and interrelationship of cytotoxicity and TBA-RS were investigated in primary short-term hepatocyte cultures from phenobarbital-pretreated rats (Göldlin and Boelsterli 1991a, b). Cocaine induced lipid peroxidation and LDH release in a time and concentration-dependent manner which could be markedly exacerbated by depletion of hepatocellular GSH with diethyl maleate. Deferoxamine, an iron chelator which inhibits formation of reactive hydroxyl radicals from superoxide anions and hydrogen peroxide, reduced both TBA-RS formation and cytotoxicity (Fig. 2). However, TPGS, a hydrosoluble alpha-tocopherol ester, had no protective effect against cytotoxicity while totally inhibiting the formation of TBA-RS (Fig. 2). Collectively, these results indicate that reactive oxygen species are involved in cocaine-induced hepatotoxicity. However, the hepatocellular damage is likely not to be initiated by the sequelae of membrane lipid peroxidation but rather by direct interaction of the reactive oxygen species with other critical targets.

Conclusions and perspectives

Biomechanistic studies on cocaine-induced hepatotoxicity have considerably increased our understanding of the interrelationship between the formation of reactive cocaine intermediates and acute lethal hepatocyte injury. The *N*-oxidative pathway of cocaine metabolism, mainly catalyzed by specific P-450 isoforms, is involved in the formation of reactive oxygen species ultimately leading to the killing of the hepatocytes. However, the present knowledge about the contribution of other biomechanisms, such as irreversible protein-adduct formation or oxidation of critical protein thiols, is still too incomplete for a definite understanding of the development of cocaine-induced hepatotoxicity. The elusiveness of clear mechanistic pathways of cocaine-induced hepatocellular injury precludes a simple explanation based on one molecular event and suggests the presence of a complex interaction of a variety of cellular alterations (Fig. 3).

Many studies that attribute lipid peroxidation, covalent binding to hepatocellular proteins, or depletion of cellular non-protein or protein thiols as related to cocaine-induced hepatic toxicity have failed to provide clear evidence for a causal relationship between these biochemical alterations and cell viability. Many of these biochemical processes could be a consequence rather than the cause of the initial damage and thus represent epiphenomena. For example, cocaine-mediated lipid peroxidation has emerged as being dissociated from other oxidative effects that are more likely to be linked with cytotoxicity (Fig. 2). Similarly, irreversible (covalent) interaction of cocaine intermediates with hepatocellular proteins may rather mirror the rate of formation of reactive intermediates than actually be mechanistically linked with the promotion of cell killing. Although a quantitative correlation between the extent of cocaine-protein adducts and the manifestation of cocaine-mediated cytotoxicity has been well established both in vivo and in vitro, the toxicological significance of the covalent interaction with selective hepatocellular proteins remains unclear.

The qualitative nature of the target molecules for both electrophilic drug intermediates and for reactive oxygen species is an important determinant in the expression of hepatocellular toxicity. Unfortunately, the paucity of knowledge about the molecular targets of irreversible binding of cocaine intermediates limits our ability to under-

stand the role of covalent binding as a possible mechanism in the pathogenesis of cocaine-induced hepatotoxicity. Similarly, the putative role of the oxidative injury of critical cellular thiols remains speculative so long as the identities of th critical targets for cocaine-induced oxidative damage are not known. Clearly, immunochemical analyses of protein-cocaine adducts will be required in order to characterize and identify the molecular target proteins as successfully shown for other hepatotoxic drugs (Bartolone et al. 1988). While it is tempting to speculate that well known secondary effects, such as changes in Ca^{2+} homeostasis due to covalent or non-covalent interaction with critical thiol groups of plasma membrane or mitochondrial ATPases, may be a consequence of the toxic insult elicited by reactive cocaine species, a final common pathway for chemically-induced hepatocellular toxicity including cocaine has yet to be demonstrated.

Although some molecular mechanisms begin to emerge as key events, our present knowledge of cocaine-induced hepatotoxicity is still incomplete. Nevertheless, the proposed model provides a baseline for future directions. For example, with the improved culture techniques for hepatocytes or precision-cut liver slices from various species including man, and with the availability of sensitive HPLC methods for cocaine and cocaine metabolites (Bouis et al. 1990), direct quantitative measurements of the *N*-oxidative cocaine metabolism will allow correlation with the extent of irreversible protein adduct formation and oxidative stress. This will make possible direct comparative estimations of the rate of electrophilic intermediate formation in intact hepatocytes. Furthermore, the growing number of drugs that are known to induce specific P-450 isoforms including the P-450IIB subfamily will extend our knowledge about possible drug interaction and modulating factors of cocaine bioactivation. Specifically, elucidation of the mechanisms of how ethanol potentiates cocaine hepatotoxicity will eventually lead to more precise estimates of high risk subgroups in the human population as the prevalence rates of concurrent and simultaneous use of alcohol and cocaine are high (Grand and Harford 1990). In addition, structure-activity analyses of cocaine analogs have been successfully used to delineate the functional role of the molecular substructures in cocaine pharmacology and toxicity (Thompson et al. 1979; Freeman and Harbison 1981b; Basmadjian et al. 1990; Boja et al. 1990). Similarly, drug congeners featuring differential toxicologic profiles could be used to dissect those biochemical processes in hepatocytes that are causally related to cytotoxicity from those that are not related to cell injury. Finally, the determination of the relative stability of cocaine metabolites in intracellular and extracellular compartments may have implications for mechanistic analyses of cocaine-induced target organ toxicity other than the liver, since the optimal degree of reactivity of a metabolite determines the range of interaction with sites critical for viability (Monks and Lau 1988). For example, direct toxic effects of cocaine have been demonstrated in cultured rat cardiomyocytes (Welder et al. 1988, 1991), but the significance of cocaine intermediates in the development of cocaine-induced cardiotoxicity has not been studied so far.

The combination of functional toxicity studies using intact cells with target molecule identification and characterization as well as the application of immunochemical and molecular techniques will be required to further illuminate our understanding of the role of the biochemical and molecular events eliciting hepatocellular necrosis and their temporal interaction during cocaine-mediated hepatotoxicity.

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