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Reductive metabolism of the sanguinarine iminium bond by rat liver preparations

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Abstract:
Background: Sanguinarine (SA) is a quaternary benzo[c]phenanthridine alkaloid that is mainly present in the Papaveraceae family. SA has been extensively studied because of its antimicrobial, anti-inflammatory, antitumor, antihypertensive, antiproliferative and antiplatelet activities. Metabolic studies demonstrated that SA bioavailability is apparently low, and the main pathway of SA metabolism is iminium bond reduction resulting in dihydrosanguinarine (DHSA) formation. Nevertheless, the metabolic enzymes involved in SA reduction are still not known in detail. Thus, the aim of this study was to investigate the rat liver microsomes and cytosol-induced SA iminium bond reduction, and to examine the effects of cytosol reductase inhibitors on the reductive activity.
Methods: DHSA formation was quantified by HPLC. The possible enzymes responsible for DHSA formation were examined using selective individual metabolic enzyme inhibitors.
Results: When SA was incubated with liver microsomes and cytosol in the absence of NAD(P)H, DHSA, the iminium bond reductive metabolite was formed. The reductase activity of the liver microsomes and cytosol was also enhanced significantly in the presence of NADH. The amount of DHSA formed in the liver cytosol was 4.6-fold higher than in the liver microsomes in the presence of NADH. The reductase activity in the liver cytosol was inhibited by the addition of flavin mononucleotide and/or riboflavin. Inhibition studies indicated that menadione, dicoumarol, quercetin and 7-hydroxycoumarin inhibited rat liver cytosol-mediated DHSA formation in the absence of NADH. However, only menadione and quercetin inhibited rat liver cytosol-mediated DHSA formation in the presence of NADH.
Conclusions: These results suggest that the SA iminium bond reduction proceeds *via* two routes in the liver cytosol. One route is direct non-enzymatic reduction by NAD(P)H, and the other is enzymatic reduction by possible carbonyl and/or quinone reductases in the liver cytosol.

Key words:
sanguinarine, reductive metabolism, quinone reductases, dihydrosanguinarine

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Introduction

Sanguinarine (SA) is a quaternary benzo[c]phenanthridine alkaloid that is mainly present in the Papaveraceae family, which includes *Sauguinaria Canadensis* L. rhizomes, *Chelidonium majus* L. roots, the aerial portion of *Macleaya cordata* and *Argemone mexicana* L. seeds [27, 38]. SA has been extensively studied because of its antimicrobial, anti-inflammatory [24], antitumor [36], antihypertensive [26], antiproliferative [2, 3] and antiplatelet [17] activities. Thus, SA is currently used in human and veterinary medicine as a bioactive component of anti-plaque preparations and in animal husbandry as the feed additive Sangrovit [12, 22]. These SA activities are linked to the equilibrium between quaternary cation and alkanolamine forms (Fig. 1) [5]. The conversion of the iminium ion to the alkanolamine improves SA lipophilicity, which may result in increased alkaloid bioavailability [9, 34]. Because of the iminium bond, SA can react with nucleophilic and anionic moieties of amino acids in biomacromolecules [32]. Furthermore, formation of a molecular complex of SA with DNA by intercalation was described [35].

In early studies, benzo[c]acridine (BCA) was described as the "green-fluorescent compound" SA metabolite *in vivo* [14]. After SA administration, BCA was found in rat, cat and monkey plasma and milk. Another metabolic study also demonstrated that BCA was excreted in urine and feces [37]. These studies suffer from several weaknesses: (1) BCA was detected by thin layer chromatography (TLC). (2) The standard BCA solution was not analyzed by TLC (3).

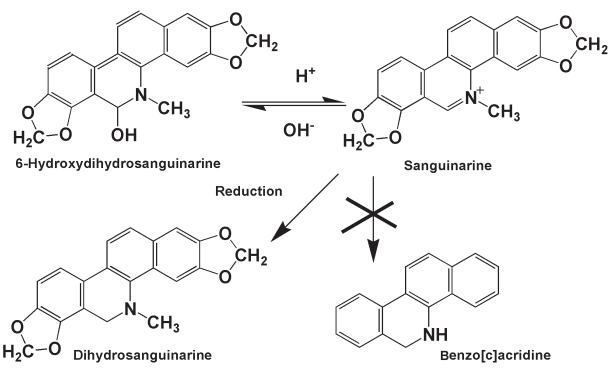

Fig. 1. Equilibrium between the sanguinarine iminium ion alkanolamine forms and the conversion of sanguinarine to dihydrosanguinarine. Sanguinarine is reduced to dihydrosanguinarine but is not metabolized to benzo[c]acridine *in vitro* and *in vivo*

The interpretation of the green-fluorescent spot as BCA was not supported by any direct structural evidence [10]. However, two recent publications had a different conclusion. Psotova et al. studied SA metabolism in rats using high-performance liquid chromatography-mass spectrometry (HPLC/MS). Higher dihydrosanguinarine (DHSA) concentrations were found in both plasma and the liver compared with SA. Neither SA nor DHSA were detected in the urine. BCA was not detected in the rat urine, plasma or liver [30]. The first report on SA pharmacokinetics and disposition further confirmed that BCA was not a SA metabolite because it was not detected in the plasma, liver or urine [39]. Pharmacokinetic results for SA demonstrated that SA as well as its main metabolite DHSA are relatively quickly cleared from the body with a $t_{1/2}$ of 3 h for both compounds. Also, the SA and DHSA C_{max} (ng/ml) was 192.3 and 545.9, respectively. After a single oral [^{3}H]SA dose, more than 42% of the ingested radioactivity was present in the gastrointestinal tract [39]. These results demonstrated that SA bioavailability is apparently low and the main SA metabolism pathway is iminium bond reduction resulting in DHSA formation (Fig. 1).

Recently, *in vitro* metabolism studies demonstrated that SA inhibited cytochrome P4501A (CYP1A1) enzyme catalytic activity [19, 41]. However, SA in submicromolar concentrations had no effects on CYP1A expression in HepG2 cells [6, 46]. SA-induced cytotoxicity in HepG2 cells and/or primary rat hepatocytes was attenuated in cultures that were pretreated with aryl hydrocarbon receptor (AhR) activators such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), 3-methylcholanthrene (3-MC) and β-naphthoflavone (BNF) [11, 41]. The primary explanation for these effects was the involvement of TCDD-inducible metabolic enzymes in SA metabolism/cytotoxicity, which were induced by TCDD, especially the cytosolic reductases and phase II enzymes [10, 15]. Recently, Deroussent et al. demonstrated that CYP1A1 and CYP1A2 were only involved in oxidative SA metabolism using eight human recombinant CYPs [8]. DHSA was the main reduced derivative formed from SA in the rat liver microsomes and CYP-expressing cells. However, the metabolic enzymes that are involved in SA reduction are still not known in detail. In the present study, the SA iminium bond reduction by rat liver microsomes and cytosol was investigated. Furthermore, the effects of cytosolic reductase inhibitors on SA reduction were examined.

Materials and Methods

Chemicals

SA and DHSA were obtained from Hunan Meikeda Co. (Changsha, China). NADPH and NADH were purchased from Roche Chemical Co. (Beijing, China). Menadione, 7-hydroxycoumarin, and flavin mononucleotide (FMN) were purchased from Sigma Chemical Co. (St. Louis, USA). Quercetin and dicoumarol were from the National Institutes for Food and Drug Control (Beijing, China). Riboflavin (RIB) was purchased from China National Pharmaceutical Group Corp. (Shanghai, China). HPLC-grade acetonitrile was obtained from Fisher Chemical Co. (New Jersey, USA). Water was purified using a Heal Force Easy system (Shanghai, China). All of the other chemicals and reagents used were of the highest analytical grade.

Animals

Four male Sprague-Dawley (SD) rats (eight weeks old) weighing approximately 280–300 g were obtained from the Hunan SLRC Laboratory Animal Center (Changsha, China). All of the rats were kept under standard environmental conditions and fed with water and food *ad libitum*. Food was withdrawn the night before experiments. The animal protocol was approved by the Ethical Committee for Animal Care and Use at the Hunan Agricultural University.

Tissue subcellular fraction preparation

Rats were exsanguinated after a 12 h fast, after which their livers, hearts, lungs, kidneys, brains, spleens, testes, duodenums, ileums, jejunums and colons were rapidly removed. These tissues were washed immediately with ice-cold 0.15 M KCl to remove blood. The tissues were then blotted with filter paper and stored at –70°C until use.

Tissue microsomes and cytosol were isolated using the calcium precipitation method [13]. Tissues were minced and washed two times with ice-cold 0.15 M KCl followed by homogenization with two volumes of 0.05 M Tris-HCl buffer (pH 7.4, containing 0.25 M sucrose). The homogenate was centrifuged at 10,000 × g for 20 min. The supernatant was taken, 88 mmol/l $CaCl_2$ (10% v/v) was added, and the supernatant was centrifuged again at 27,000 × g for 15 min. Pooled cytosolic fractions were obtained from the supernatants. Pooled microsome pellets were resuspended in a 0.05 M Tris-HCl buffer. The microsomes and cytosol were stored at –70°C until use for incubation assays. An aliquot of each subcellular fraction was used to determine protein content using the Bradford method with a bovine serum albumin standard. Microsomes and cytosol were heated in a boiling water bath for 5 min to prepare boiled microsomes and cytosol.

Microsomal DHSA metabolite assay

The incubation mixture (200 µl) consisted of SA, 2 mM NADH or NADPH, and microsomes (0.4 mg protein) in 0.05 M Tris-HCl buffer (pH 7.4). The reaction mixture was preincubated for 2 min at 37°C, and the reaction was started by NADH or NADPH addition and continued for 20 min at 37°C. After incubation, the mixture was terminated by the addition of 200 µl of ice-cold acetonitrile. An aliquot (20 µl) of the clear supernatant was analyzed by high-performance liquid chromatography (HPLC). Using rat liver microsomes in the presence of NADPH, DHSA formation was linear over a range of 0.1 to 0.5 mg protein.

Cytosolic DHSA metabolite assay

The incubation mixture (200 µl) consisted of SA, 2 mM NADH or NADPH, and cytosol (0.4 mg protein) in 0.05 M Tris-HCl buffer (pH 7.4). The reaction mixture was preincubated for 2 min at 37°C, and the reaction was then started by SA addition and continued for 20 min at 37°C. After incubation, the mixture was stopped as described earlier. Using rat liver cytosol in the presence of NADH, DHSA formation was linear over a range of 0.1 to 0.5 mg protein.

SA reduction with NAD(P)H

The incubation mixture (200 µl) consisted of SA, 2 mM NADH or NADPH, and 100 µM FMN and/or RIB in 0.05 M Tris-HCl buffer (pH 7.4). The reaction mixture was preincubated for 2 min at 37°C, and the reaction was started by SA addition and continued for 20 min at 37°C. The mixtures were performed as described for DHSA metabolite assay above.

Inhibition experiments

The incubation mixtures for inhibition studies contained 2 mg/ml cytosolic protein, 10 μM SA and 100 μM inhibitor in the absence or presence of 2 mM NADH. The inhibitors were menadione (aldehyde oxidase, carbonyl reductase), 7-hydroxycoumarin (xanthine oxidase), dicoumarol (quinone oxidoreductase 1) and quercetin (quinone oxidoreductase 2, carbonyl reductase). With the exception of dicoumarol, which was dissolved in DMSO, all of the other inhibitors were dissolved in methanol, and the organic solvent content did not exceed 1% (v/v) in the incubation.

The inhibitor-treated sample values were compared only with the control SA values, and the inhibition values were expressed as a percentage of the control values. Liver cytosol was incubated with various inhibitor concentrations (1–200 μM) and 10 μM SA to determine the IC_{50} values. All of the incubations and sample preparations were performed as described for DHSA metabolite assay above.

HPLC for DHSA determination

DHSA was quantified using a Shimadzu LC- 2010AHT system. The HPLC system (Shimadzu Co., Kyoto, Japan) was equipped with a LC-10ADvp pump, a SIL-10ADvp autosampler, and a SOD-10ADvp detector. Separation of SA and its reduced metabolite DHSA was performed on a VP-ODS Hypersil column (4.6 × 250 mm I.D.; particle size 5 μm; Shimadzu Co, Japan). The mobile phase consisted of a 37/63 (v/v) mixture of acetonitrile and water. The injection volume was 20 μl and the flow rate was 1 ml/min. The sample chamber in the autosampler was maintained at ambient temperature, while the column temperature was set to 35°C. The detection wavelength was set to 285 nm. The DHSA standard curve was linear in the concentration range from 0.025 to 1 μM. The DHSA inter- and intra-assay coefficients of variation were 2.3 and 6.2% at 0.05 μM, respectively. The lower limit of quantification was 0.025 μM.

Data analysis

All of the reported data were represented as the means ± SD unless stated otherwise. The metabolic rates were expressed in pmol metabolites formed per min per mg protein (pmol/(min × mg)). The inhibition potency (IC_{50}) values were determined by the Sigma Plot 10.0 (SPSS Inc., Chicago, IL) wherever appropriate using the following equation:

$$V = V_0/[1 + I/IC_{50}]^a$$

where V_0 is the uninhibited velocity, V is the observed velocity, ^{a} is the slope factor, and I is the inhibitor concentration.

The analysis of statistical significance (* $p < 0.05$) was performed. A paired-sample *t* test was used to compare the incubation and control groups.

Results

SA reduction by rat liver microsomes and cytosol

Representative HPLC chromatograms of SA incubated with rat liver microsomes and cytosol are displayed in Figure 2. When SA was incubated with rat

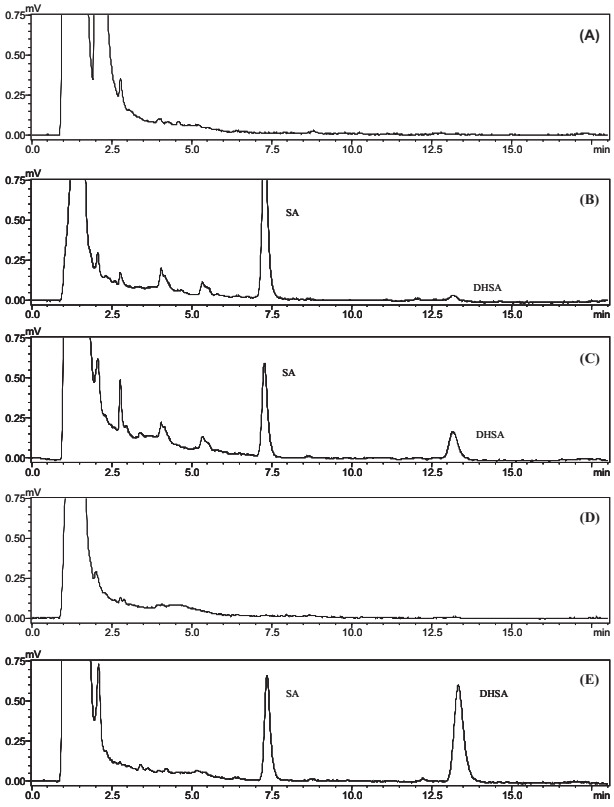

Fig. 2. Representative HPLC chromatograms of control liver microsomes (**A**), SA (10 μM) was incubated with liver microsomes in the absence of NADPH (**B**), SA (10 μM) was incubated with liver microsomes in the presence of NADPH (**C**), control liver cytosol (**D**), SA (10 μM) was incubated with liver cytosol in the absence of NADPH (**E**). SA was incubated with 2 mg/ml liver microsomes or cytosolic protein in the absence or presence of NADPH for 20 min

Tab. 1. SA iminium bond reduction by native or boiled rat liver microsomes and cytosol. SA (10 μM) was incubated with 2 mg/ml liver microsome or cytosolic protein in the absence or presence of NAD(P)H for 20 min. The DHSA formed was determined by HPLC. Each value is the mean ± SD from three experiments

Addition	DHSA formed (pmol/(min × mg))			
	Cytosol	Boiled cytosol	Microsomes	Boiled microsomes
None	15.9 ± 4.1	N.D.*	1.2 ± 0.4	N.D.*
NADH	26.1 ± 4.3	12.4 ± 1.1*	5.8 ± 0.8	10.9 ± 1.5*
NADPH	11.8 ± 1.0	7.8 ± 0.7*	2.1 ± 0.3	8.7 ± 0.5*

* Significantly different compared with the native group

liver microsomes in the absence of NADPH, a small amount of the reductive metabolite DHSA was detected. SA reduction in liver microsomes was enhanced significantly by NADPH addition. SA and DHSA retention times were 7.3 and 13.5 min, respectively. When SA was incubated with rat liver cytosol in the absence of NAD(P)H, a large amount of DHSA was also detected. These peaks were not observed in the control chromatogram, which was incubated without SA addition.

Iminium bond reductase activity of rat liver microsomes and cytosol

The liver microsomes and cytosol all exhibited iminium bond reductase activity toward DHSA in the absence or in the presence of NAD(P)H (Tab. 1). DHSA formation by the liver microsomes and cytosol proceeded at a rate of approximately 1.2 and 15.9 pmol/(min × mg) in the absence of NAD(P)H, respectively. Liver microsome and cytosol reductase activity was also enhanced significantly in the presence of NADH. The amount of DHSA formed in the liver cytosol was 4.6-fold higher than in the liver microsomes in the presence of NADH. However, DHSA formation by liver microsomes and cytosol in the presence of NADPH was 2.7-fold and 2.2-fold lower, respectively, than what was observed in the presence of NADH. These observations indicated that cytosolic enzymes are highly likely to be involved in SA reduction in the rat liver. In this case, NADH was more effective than NADPH as an electron donor for the SA iminium bond reduction. Therefore, all of the subsequent metabolism studies were performed with the liver cytosol.

Tab. 2. SA iminium bond reduction by NADH and/or cofactors in the absence of microsomes and cytosol. Incubations were performed with NADH and/or cofactors for 20 min. The DHSA formed was determined by HPLC. Each value is the mean ± SD from three experiments

Addition	DHSA (pmol/l)
NADH	260 ± 30
NADPH	220 ± 10
Riboflavin	ND
FMN	ND
Riboflavin, FMN	ND
NADH, riboflavin	170 ± 10
NADH, FMN	200 ± 30
NADH, riboflavin, FMN	210 ± 40

The NADH-linked rat liver cytosol reducing activity was not stimulated under hypoxic conditions (data not shown). Thus, the SA iminium bond reduction was independent of oxygen. When liver microsomes and cytosol were boiled, reductase activity was abolished in the absence of NAD(P)H (Tab. 1). However, SA formation was observed in the boiled cytosol in the presence of NADPH or NADH. Further, SA iminium bond reduction was examined in the absence of microsomes and cytosol. SA was reduced to DHSA in the presence of NADPH or NADH without microsomes or cytosol. However, the reductive activity was not stimulated in the presence of NADH and riboflavin and/or FMN (Tab. 2). These results suggest that SA was also directly reduced to DHSA by NADH in the absence of protein.

The liver cytosol reductase activity was completely inhibited by the addition of FMN and/or RIB. The rat liver cytosol NADH-dependent reductase activity was also inhibited by the addition of these cofactors (Fig. 3). Of the tissues examined, the reductase activity in the kidney and heart cytosol was 8- and 11-fold lower in the absence of NADH than in the liver cytosol, re-

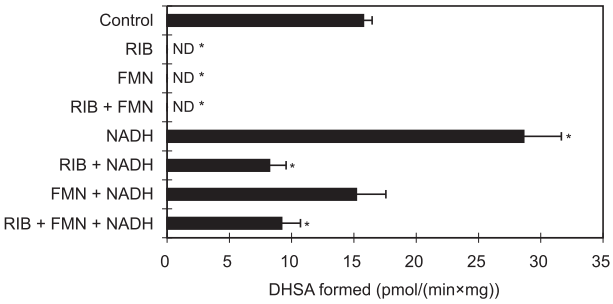

Fig. 3. Reduction of SA to DHSA by rat liver cytosol in the presence of cofactors. SA (10 μM) was incubated with 2 mg/ml liver cytosolic protein in the presence of cofactor for 20 min. The DHSA formed was determined by HPLC. ND, not detected. Each value is the mean ± SD from three experiments. * Significantly different compared with the control

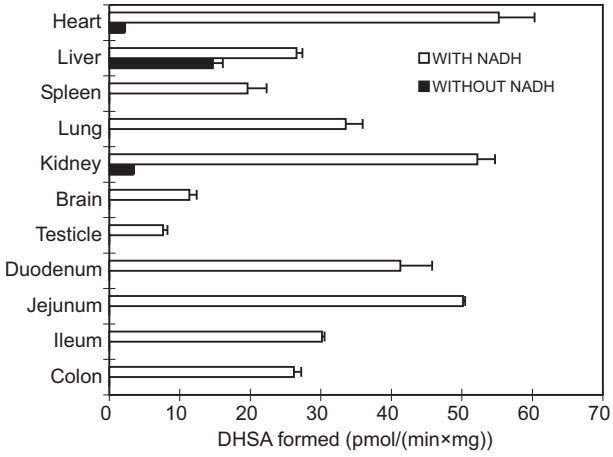

Fig. 4. Reduction of SA to DHSA by rat tissue cytosols. SA (10 μM) was incubated with 2 mg/ml cytosolic protein in the absence or presence of NADH for 20 min. The DHSA formed was determined by HPLC. Each value is the mean ± SD from three experiments

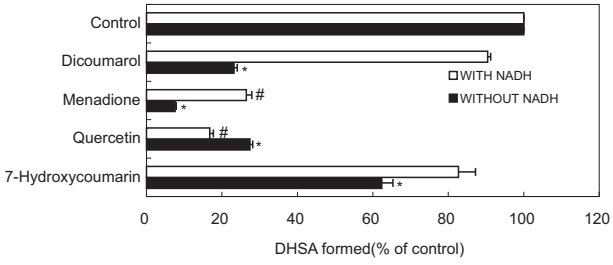

Fig. 5. Effect of some inhibitors on the SA iminium bond reduction to DHSA by rat liver cytosol. SA (10 μM) was incubated with 2 mg/ml cytosolic protein in the absence or presence of inhibitors (100 μM) for 20 min. The results are reported as a percentage of control activity. Mean control values (in the absence of inhibitors) for rat liver cytosol-mediated DHSA formation in the absence of and the presence of NADH were 15.9 and 26.3 pmol/(min × mg), respectively. The DHSA formed was determined by HPLC. Each value is the mean ± SD from three experiments. * Significantly different compared with the control without NADH. # Significantly different compared with the control with NADH

spectively (Fig. 4). The other tissues cytosol did not exhibit the reductase activity in the absence of NADH. The cytosol from the other tissues all exhibited iminium bond reductase activity toward DHSA in the presence of NADH because of direct NAD(P)H and SA reduction. These observations suggest that the SA iminium bond reduction is catalyzed by some flavinprotein reductases in the liver cytosol.

Effect of inhibitors on iminium bond reduction

The effects of inhibitors on DHSA formation in the rat liver cytosol are demonstrated in Figure 5. It was determined that the rat liver cytosol iminium bond reductase activity was inhibited by the addition of menadione, dicoumarol, quercetin and 7-hydroxycoumarin in the absence of NADH. However, only menadione and quercetin inhibited DHSA formation by the rat liver cytosol in the presence of NADH.

For determining the inhibitor IC_{50} values, the rat liver cytosol was incubated with various inhibitor concentrations (0.5–200 μM) and 10 μM SA. The effects of the inhibitors on DHSA formation in the absence and in the presence of NADH are demonstrated in Figure 6. It was indicated by all of the plots that DHSA activity was inhibited in a concentration-dependent manner. Menadione and quercetin treatment inhibited DHSA formation by the liver cytosol in the absence of NADH with IC_{50} values of 7.6 μM and 41.3 μM, respectively, and in the presence of NADH with IC_{50} values of 46.8 and 4.3 μM, respectively.

Discussion

The conversion of SA to DHSA is an important detoxification pathway in animals and humans. Previous studies demonstrated that no toxic response was found in animals after oral DHSA administration, and that DHSA was less cytotoxic than SA [40, 43]. However, the mechanism of SA reduction in animals remains unclear. In this study, the SA iminium bond reduction in the rat liver microsomes and cytosol was investigated. This study demonstrated that the SA iminium bond was reduced by rat liver microsomes and cytosol in the absence of NAD(P)H. However, the amount of DHSA formed in liver microsomes was ex-

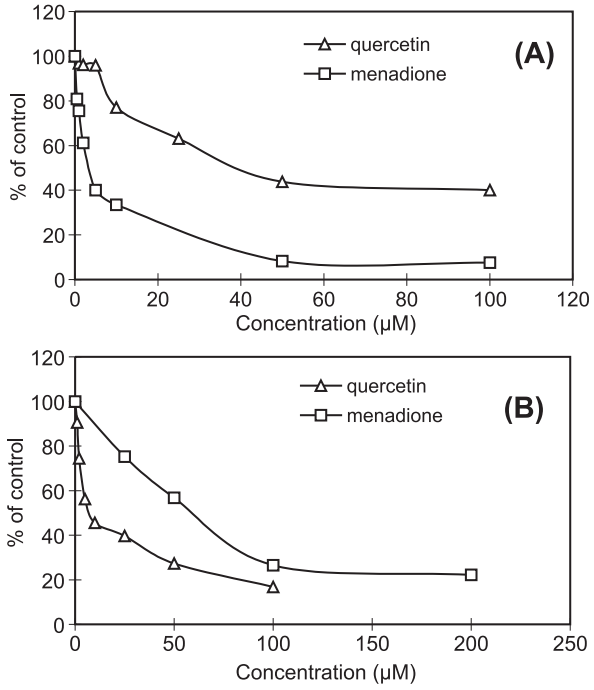

Fig. 6. Effects of inhibitors on the SA iminium bond reduction to DHSA by rat liver cytosol in the absence of NADH (**A**) and in the presence of NADH (**B**). SA (10 μM) was incubated with 2 mg/ml rat liver cytosolic protein in the absence or presence of inhibitors for 20 min. The results are reported as a percentage of the control activity. Mean control values (in the absence of inhibitors) for DHSA formation by rat liver cytosol in the absence and in the presence of NADH were 16.4 and 24.5 pmol/(min × mg), respectively. The DHSA formed was determined by HPLC. Each value is the mean ± SD from three experiments

tremely low compared with the liver cytosol. Higher SA concentrations (above 10 μM) caused NADPH-dependent reduction to slow down, as reported by Weiss et al. [45]. Therefore, NADH was more effective than NADPH as an electron donor for the SA iminium bond reduction. Based on the present results, SA reduction is controlled reaction by native liver enzyme preparations in the absence of cofactors. Moreover, kidney and heart cytosol also exhibited reductase activity in the absence of NADH.

DHSA formation by reducing enzyme cofactors such as NADH and NADPH has already been demonstrated [16, 23]. Recently, SA metabolism by NADH reportedly produced two products with blue fluorescence. The major product was an anion, which was more stable than the uncharged dihydro form [28]. DHSA exhibited no changes between pH 5 and 12, which provides an explanation for the relative DHSA stability at neutral or alkaline pH [16]. However, additional physiologically important reducing agents (such as glutathione and L-ascorbic acid) were unable to reduce SA, as reported by Kosina et al. [21]. The current study demonstrated that SA was not reduced by boiled microsomes and cytosol in the absence of NAD(P)H. However, boiled microsomes and cytosol also exhibited SA reductase activity in the presence of NAD(P)H. SA was also directly reduced to DHSA by NADH without protein (Fig. 7). Thus, the SA iminium bond reduction is dependent on the reducing agent NAD(P)H.

In the present study, DHSA formation was in the presence of NADH not stimulated by the addition of flavin. Moreover, RIB and FMN could inhibit DHSA formation in the absence of microsomes and cytosol. In liver cytosol, RIB and FMN could also inhibit DHSA formation in the absence and presence of NAD(P)H. Flavin reportedly can be non-enzymatically reduced by NADH or NADPH [33]. Flavin reduction is also catalyzed by NAD(P)H-flavin reductases in liver cytosol. The flavin moieties are likely to be involved in transferring electrons from flavin reductase enzymes to the substrate, but presumably these electrons are derived indirectly from NAD(P)H. Thus, the flavin-mediated DHSA inhibition is medi-

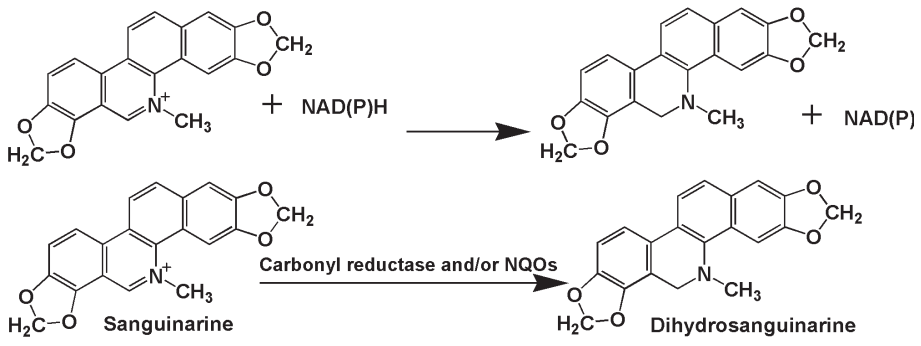

Fig. 7. Reduction of the sanguinarine iminium bond by rat liver cytosolic NAD(P)H and reductases

ated by preventing the supply of reducing equivalents to the substrate SA.

Sanguinarine reductase was found in *Eschscholzia* leaf and root protein extracts and was isolated from suspended cultured cells [45]. SA occurs in the oxidized form only on the outside of plant cells and as DHSA on the inside. Sanguinarine reductase initiates a recycling process that allows cytotoxic benzophenanthridine presentation as phytoalexins at the cellular surface and concomitantly prevents self-intoxication. Based on the x-ray structure of a close homolog, the enzyme displayed close structural homologies with domains of NAD(P)-dependent human 17β-hydroxysteroid dehydrogenase (17HSD). 17HSD belongs to the aldo-keto reductase family (AKRs), which catalyze aldehyde and ketone moiety reduction [18]. Current findings suggest that some reductases do play role in SA reduction, for which the electrons are derived from NAD(P)H. Reductases that are involved in reductive metabolism including AKRs, carbonyl reductase, quinone reductases, aldehyde oxidase (AO), and xanthine oxidase (XO) have the most well-known roles [20, 31]. Among these enzymes, carbonyl reductase and quinone reductases (NQOs), which includes NAD(P)H: quinone oxidoreductase 1 (NQO1) and its homologue NRH, and quinone oxidoreductase 2 (NQO2) catalyze metabolic quinone detoxification and protects cells from redox cycling, oxidative stress and neoplasia [25]. Thus, we conducted a preliminary study to examine which reductases are involved in the SA detoxification pathway in animals.

In the present study, dicoumarol and quercetin, which are selective NQO1 and NQO2 inhibitors, respectively [25], were used to investigate the role of the two enzymes in SA reduction. In addition, quercetin selectively inhibited carbonyl reductase. The two inhibitors inhibited SA reduction in the absence of NADH, suggesting that NQO1 and NQO2 are the enzymes that catalyze SA reduction. As expected, quercetin also inhibited SA reduction in the presence of NADH. However, dicoumarol treatment did not inhibit SA reduction in the presence of NADH. Further, menadione, a specific substrate of NQO2 and carbonyl reductase [4], also inhibited SA reduction in the absence and presence of NADH. The XO inhibitor 7-hydroxycoumarin [7] had very little effect on DHSA formation in rat liver cytosol in the absence of NADH, but it did not in the presence of NADH. These data indicated that XO is not involved in SA reduction. This observation is consistent with a previous report [28]. These data suggested that carbonyl reductase and/or NQOs may be responsible for the cytosolic SA iminium bond reduction. The mechanism for the rat liver cytosol-mediated SA iminium bond reduction to form DHSA is proposed in Figure 7.

This study demonstrates that SA iminium bond reduction is likely to be catalyzed by cytosolic enzymes. We also propose that carbonyl reductase and/or NQOs may be involved in SA reduction. Recently, Vrba et al. reported that SA activated the transcription factor Nrf2 and induce Nrf2 target gene expression [1, 42]. AhR activators may be metabolized by CYP1A1/2, and the reactive metabolites then induce Nrf2-regulated cytoprotective gene expression, including the gene encoding NQO1 [29]. NQO2 expression is also regulated by Nrf2 [45]. Therefore, SA could modulate the expression of these reductases that are involved in its conversion to DHSA. Future research must be done to determine what enzymes catalyze SA reduction using recombinant carbonyl reductase and NQOs and molecular biology methodologies. The results may have implications for understanding why SA toxicity is attenuated by AhR activator pretreatment, because cytosolic reductases are induced by AhR activators.

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