PHARMACOKINETICS, PHARMACODYNAMICS AND DRUG METABOLISM

Inhibition of NADPH–Cytochrome P450 Reductase by Tannic Acid in Rat Liver Microsomes and Primary Hepatocytes: Methodological Artifacts and Application to Ischemia–Reperfusion Injury

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ABSTRACT: Tannic acid (TA) inhibits nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P450 reductase (CPR) activity, which is measured by reduction of cytochrome *c*, in rat liver microsomes (RLMs). In the current study, we noticed that TA directly reduces cytochrome *c* in the absence of microsomes, thus confounding the CPR activity assay. A method is presented that measures CPR activity in the presence of TA by subtracting the cytochrome *c* reduction in the absence of NADPH (TA effect) from that in the presence of NADPH (TA plus CPR effect). The method was used to determine the inhibitory effect of TA in RLMs, recombinant CPR enzyme, and primary hepatocytes. Additionally, application of TA in a study of role of CPR in a primary rat hepatocyte model of ischemia–reperfusion (IR) was investigated. TA showed concentration-dependent, complete inhibition of CPR with half maximal inhibitory concentration (IC_{50}) values of 58.2 μM in RLMs and 54.6 and 275 μM in primary rat hepatocytes in the absence and presence of serum in the medium, respectively. Additionally, inhibition of CPR by TA was associated with a significant reduction in reactive oxygen species and cell death after IR injury. These data may be useful in future studies using TA as an inhibitor of CPR in microsomes and primary hepatocytes. © 2011 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:3495–3505, 2011

Keywords: tannic acid; NADPH–Cytochrome P450 reductase; microsomes; hepatocytes; inhibition; protein binding; simulated ischemia–reperfusion injury; cell culture; drug metabolizing enzymes

INTRODUCTION

Nicotinamide adenine dinucleotide phosphate (NADPH)–cytochrome P450 reductase (CPR) is a flavoprotein that is highly abundant in the endoplasmic reticulum of the liver. A major function of CPR is to donate electrons to cytochrome P450 (P450) monooxygenases, acting as a rate-limiting step for P450-catalyzed reactions.[1] CPR has also been reported to generate reactive oxygen species (ROS) by both direct[2,3] and indirect (via P450)[4] mechanisms, potentially causing oxidative stress and DNA damage.[5] However, the effects of CPR on ROS generation and its contribution to oxidative stress in disease states are not known at this time.

To study the potential role of CPR in disease models, pharmacological and/or transcriptional/translational approaches to manipulate the content and activity of CPR both *in vitro* and *in vivo* are needed. We recently[6] reported an antisense approach to inhibit CPR in cell culture and *in vivo* in rats. For chemical inhibition, Baer-Dubowska et al.[7] showed that tannic acid (TA), a well-known naturally occurring polyphenol, inhibits CYP1A1, CYP1A2, and CY2B enzymes in mouse liver microsomes. Further work by the same group[8] showed that TA also reduces the activities of some of these P450 enzymes *in vivo* in mice. More recently, Yao et al.[9] reported that TA inhibits relatively a large number of P450 isoenzymes nonselectively, both in rat and human liver

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microsomes. These authors also showed that TA is an inhibitor of CPR with IC_{50} values of 11.8 and 17.4 μM in rat and human liver microsomes, respectively. Therefore, they suggested that the nonselective inhibitory effects of TA on P450 isoenzymes might be due, at least in part, to the TA's ability to inhibit CPR.

On the basis of study of Yao et al.,[9] we decided to use TA as a chemical inhibitor of CPR to test the effects of CPR inhibition on disease models of oxidative stress, such as hepatic ischemia–reperfusion (IR) injury. However, during our preliminary studies, we noticed that TA directly reduces cytochrome *c*, which is normally used for quantitation of the activity of CPR.[10–13] Therefore, measurement of the CPR activity using the cytochrome *c* assay in the presence of TA in the reaction mixture could potentially yield inaccurate results. In the present communication, we report a modified cytochrome *c* assay to accurately measure the activity of CPR in the presence of TA. Additionally, the CPR inhibitory effects of TA on the rat liver microsomes (RLMs), recombinant CPR enzyme, and primary hepatocytes are reported using the modified assay. Finally, the effects of CPR inhibition by TA on ROS generation and cell death in a simulated IR injury model using primary rat hepatocytes are described.

MATERIALS AND METHODS

Chemicals and Reagents

Tannic acid and 1-aminobenzotriazole (ABT) were obtained from Sigma–Aldrich (St. Louis, Missouri). Equine heart cytochrome *c* was purchased from Calbiochem (San Diego, California). Reduced beta NADPH was procured from Calzyme Laboratories, Inc. (San Luis Obispo, California). Waymouth's MB 752/1 medium was obtained from Invitrogen (Carlsbad, California). Recombinant rat CPR Supersomes™, which are microsomes derived from baculovirus-infected insect cells, were obtained from BD Biosciences (Bedford, Massachusetts). Fetal bovine serum (FBS) was purchased from Equitech-Bio, Inc. (Kerrville, Texas). All other chemicals were of the highest grade available and obtained from commercial sources.

Animals

Male Sprague–Dawley rats (240–320 g) were purchased from Charles River Laboratories (Indianapolis, Indiana) and housed in Institutional Animal Husbandry. Rats were allowed free access to food and water and were maintained under 12-h light/dark cycle prior to the experiments. All procedures involving animals were approved by our Institutional Animal Care and Use Committee.

Preparation of RLMs

Microsomes were prepared using differential centrifugation, as explained in detail before.[14] Final microsomal pellet was dispersed in an ice-cold buffer containing 150 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, and 50 mM Tris–HCl buffer (pH 7.4) and stored at −80°C. Microsomal protein content was estimated by Bradford assay using bovine serum albumin as standard.

Primary Rat Hepatocyte Isolation and Culture

Rats were subjected to 24 h of fasting prior to hepatocyte isolation. Hepatocytes were isolated using a hepatocyte isolation kit (Worthington Biochemical Corporation, Lakewood, New Jersey), based on the instructions of the manufacturer. After isolation, the viability of hepatocytes was determined using trypan blue exclusion assay and was exceeding 80%. Hepatocytes (7.5×10^5 cells per well) were cultured in rat tail collagen (type I)-coated six-well microtiter plates using Waymouth's MB 752/1 medium containing 10% FBS, 100 nM insulin, 100 nM dexamethasone, 100 IU/mL penicillin, and 100 μg/mL streptomycin. After 4 h of seeding, cells were washed twice with phosphate-buffered saline and fresh culture medium was added. All the experiments were performed after an overnight culture of hepatocytes.

Effects of the Length of Preincubation of TA-Treated Microsomes with Cytochrome *c* on the Apparent Microsomal CPR Activity

Previously reported CPR activity assays[10–13] using 50 μM cytochrome *c* was used for these experiments. Microsomes were incubated with or without TA (60 μM) for 10 min at 37°C. Then, the microsomes were preincubated with cytochrome *c* for 0, 3, 6, or 12 min before starting the reaction with 200 μM NADPH. The rate of cytochrome *c* reduction was monitored at a wavelength of 550 nm spectrophotometrically for 3 min at 30°C.

Direct Effects of TA on Cytochrome *c* Reduction

To evaluate the direct effects of TA on cytochrome *c* reduction, various concentrations of TA (0.2–2 μM) were added to cytochrome *c* (100 μM) in the presence and absence of NADPH (200 μM). The rate of cytochrome *c* reduction was then monitored spectrophotometrically at a wavelength of 550 nm for 3 min at 30°C. These studies were performed in the absence of RLMs. On the basis of these studies, we devised a method for measurement of the CPR activity of RLMs in the presence of TA with and without NADPH.

Measurement of Microsomal CPR Activity in the Presence of TA

Because of direct cytochrome *c* reductive activity of TA, standard protocols for measurement of microsomal CPR activity[10–13] could not be followed. Therefore, we used a modified version of the kinetic method reported before.[13] Our total reaction volume was 1 mL, consisting of TA-treated microsomes (12 μg) and oxidized cytochrome *c* (100 μM) in the presence and absence of NADPH (200 μM) in 0.3 M potassium phosphate buffer (pH 7.7). The rate of cytochrome *c* reduction was monitored at 550 nm for 3 min (30°C). After preincubation of cytochrome *c* with NADPH in buffer, the reactions were started by the addition of microsomes. The true CPR activity was then determined by subtracting the rate of cytochrome *c* reduction in the absence of NADPH from that in the presence of NADPH.

Performance of the Modified Assay in the Presence of Different Concentrations of TA in the Assay Buffer

To test that the CPR activity measured using the modified assay is independent from the TA concentration in the assay buffer, microsomal CPR activity was measured using various concentrations of microsomal protein, which results in various concentrations of TA, in the assay buffer. Microsomes (200 μg/mL) were treated with TA (60 μM) for 10 min at 37°C. Then, 60, 80, or 100 μL of the TA-treated microsomes (containing 12, 16, or 20 μg microsomal protein) were used in the assay described above, and CPR activity was determined. This design results in a constant concentration of TA (60 μM) during the preincubation while having different concentrations of TA in the final assay buffer (3.6–6.0 μM). An accurate assay is expected to show a CPR activity independent of the concentrations of TA (volume of TA-treated RLMs) in the assay buffer.

Determination of the Inhibitory Effect of TA on Microsomal and Recombinant CPR Activity

Microsomes (200 μg/mL) or recombinant rat CPR supersomes (37.5 μg/mL) were preincubated with various concentrations of TA (10–100 μM) for 10 min. Subsequently, 12 μg microsomal protein or 2.25 μg CPR supersomes (60 μL) were subjected to the assay described above. The final concentration of TA in the assay buffer was 0.6–6 μM.

Inhibitory Effects of TA on CPR Activity in Primary Rat Hepatocytes in the Presence of a Serum-Containing Culture Medium

To determine concentration–CPR inhibitory activity profile of TA, primary rat hepatocytes were treated with various concentrations of TA (0, 25, 50, 100, 200, and 500 μM) in the culture medium for 24 h. Subsequently, cells were scraped into ice-cold phosphate-buffered saline and centrifuged at 14,000*g* for 5 min to obtain a pellet. The pellet was lysed using a cell lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic acid (EGTA), 10% glycerol, 1% Triton X-100, and Sigma protease inhibitor cocktail), and total protein was measured by Bradford assay. The hepatocyte lysate was then used for the CPR activity assay. To investigate the time dependency of the TA effect, hepatocytes were treated with 250 μM TA for 0, 4, 8, 16, and 24 h in the culture medium, and the cell lysate was obtained as described above.

Inhibitory Effects of TA on CPR Activity in Primary Rat Hepatocytes in the Absence of Serum

We also studied the time and concentration dependency of the TA effect on CPR in primary rat hepatocytes in the absence of proteins using Krebs Ringer HEPES (KRH) buffer containing 115 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM HEPES (pH 7.4). For the concentration dependency study, hepatocytes were treated with various concentrations of TA (0, 10, 20, 50, 100, 200, and 500 μM) in KRH buffer for 8 h. For the time dependency study, hepatocytes were treated with 100 μM TA for 0, 2, 4, and 8 h. The cell lysates were prepared as described above and used for determination of CPR activity.

Reversibility of TA-Mediated CPR Inhibitory Response in Primary Rat Hepatocytes

In order to evaluate reversibility of TA-mediated CPR inhibition, primary rat hepatocytes were treated with 250 μM TA for 24 h in the presence of the culture medium (with serum). Subsequently, cells were washed thrice with phosphate-buffered saline, and KRH buffer (pH 7.4) was added to the cells. Hepatocytes were collected for CPR activity measurement after 0, 4, and 8 h of TA-free KRH buffer incubation.

Effects of KCN on the CPR Activities of Microsomes and Hepatocytes

The above CPR assays in the microsomes and hepatocytes were conducted in the absence of KCN. To investigate whether presence or absence of KCN affects the observed CPR activities and/or the degree of inhibition of CPR by TA, further experiments were carried out as follows. Microsomes or primary rat hepatocytes in KRH buffer were preincubated for 10 min or 8 h, respectively, at 37°C in the presence or absence of 50 μM TA. Subsequently, the microsomes and hepatocyte lysates were subjected to our modified CPR assay described above in the presence and absence of 1 mM KCN in the assay buffer.

Effects of CPR Inhibition by TA on Simulated IR Injury in Primary Rat Hepatocytes

We used a previously validated model of simulated IR injury in primary rat hepatocytes[15] to test the effects of inhibition of CPR by TA on the IR injury. Briefly, after an overnight culture, hepatocytes in 24-well microtiter plates (1.5×10^5 cells per well) were subjected to 4 h of ischemia (95% N_2:5% CO_2; $pO_2 <$ 0.2%) in a pH 6.2 KRH buffer, followed by 20 min of reoxygenation in a pH 7.4 buffer. TA at a concentration of 100 μM was added 30 min before the induction of simulated ischemia, whereas no drug was added to the control wells. To distinguish the effects of CPR inhibition from those of P450 inhibition, a third group of wells were treated with 1-ABT at a concentration of 5 mM. The inhibitors were present in the buffer during both ischemia and reoxygenation periods. Typical IR injury markers such as ROS and cell viability were measured using 5 μM 2',7'-dichlorfluorescin-diacetate (DCF-DA)[16] and 30 μM propidium iodide,[17] respectively. Briefly, ROS generation was measured by 30 min incubation of DCF-DA with the cells and fluorescence monitoring of the formation of DCF from the nonfluorescent precursor DCF-DA. Additionally, cell viability was estimated by measuring the fluorescence of propidium iodide/DNA adducts, correcting for the fluorescence of digitonin-treated cells as a measure of total number of cells, as reported before.[17] DCF fluorescence was corrected for the amount of protein in the wells.

Data and Statistical Analysis

The relationship between the TA concentrations (C) and CPR inhibitory response (I) was fitted according to the following equation using nonlinear regression analysis:

$$I = \frac{I_{max} \times C^{\gamma}}{IC_{50}^{\gamma} + C^{\gamma}} \quad (1)$$

where I_{max}, IC_{50}, and γ are maximum inhibitory effect, the C producing half of I_{max}, and the Hill coefficient, respectively. Data with two groups were analyzed using two-tailed Student's t test, whereas comparison of means from three groups was conducted using one-way analysis of variance (ANOVA), followed by Tukey's multiple comparison post-hoc test. The effect of length of preincubation on the CPR activity was analyzed using linear regression analysis. The effects of KCN and TA on the absolute CPR activities were analyzed using two-way ANOVA, followed by Bonferroni post-hoc test. A p value of less than 0.05 was considered as statistically significant. Results are expressed as mean ±SD.

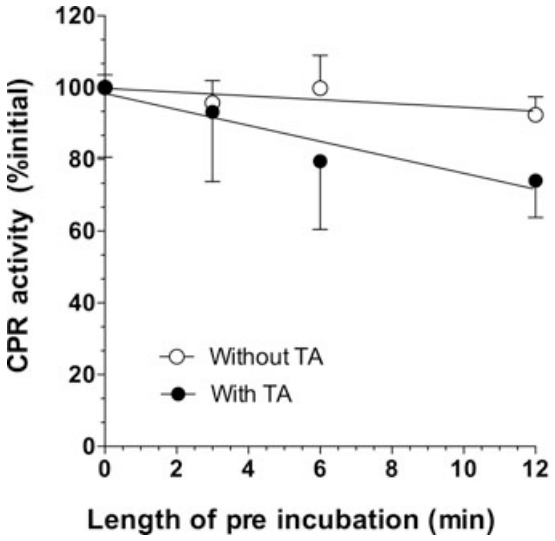

Figure 1. Effects of the length of preincubation of microsomes with cytochrome c before the start of the reaction by the addition of NADPH on the apparent microsomal CPR activity in the absence (without TA) or presence (with TA) of tannic acid in the sample. The symbols and bars represent the mean and SD values, respectively, and the lines are obtained by linear regression analysis ($n = 3$). Linear regression analysis of the CPR activity versus length of preincubation time was significant ($p < 0.05$) only in the presence of TA.

RESULTS

Effects of the Length of Preincubation of TA-Treated Microsomes with Cytochrome *c* on the Apparent Microsomal CPR Activity

The apparent CPR activity of the microsomal samples, measured by the conventional assays,[13] are shown in Figure 1 for samples preincubated with cytochrome c for various times before initiating the reactions by the addition of NADPH. As expected, the length of preincubation time did not have any significant effect on the control (without TA) microsomes. However, in the presence of TA in the sample, the apparent CPR activity significantly ($p < 0.05$, linear regression analysis) declined with an increase in the length of preincubation. This was due to a reduction of cytochrome c by TA even before the addition of NADPH, resulting in a lower concentration of the substrate (cytochrome c concentration) in the assay buffer available at the time of NADPH addition.

Direct Reduction of Cytochrome *c* by TA

Figure 2 illustrates the relationship between the concentrations of TA in the assay buffer and reduction of cytochrome c in the absence of any RLMs or CPR. As demonstrated in this figure, TA reduced cytochrome c in a concentration-dependent manner. Indeed, there is a linear relationship between the extent of

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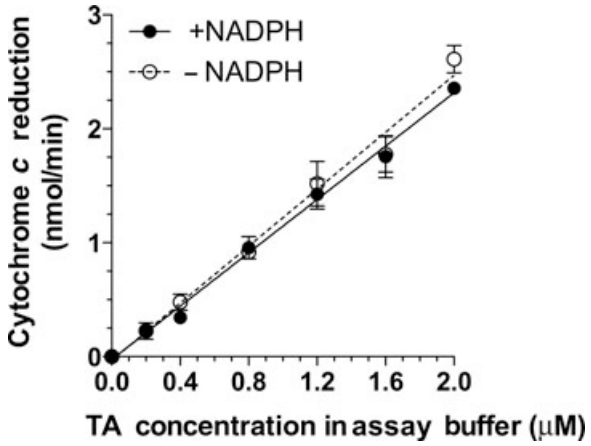

Figure 2. Direct reduction of cytochrome *c* by tannic acid (TA) in the absence or presence of NADPH in the assay buffer. The symbols and bars represent the mean and SD values, respectively ($n = 3$).

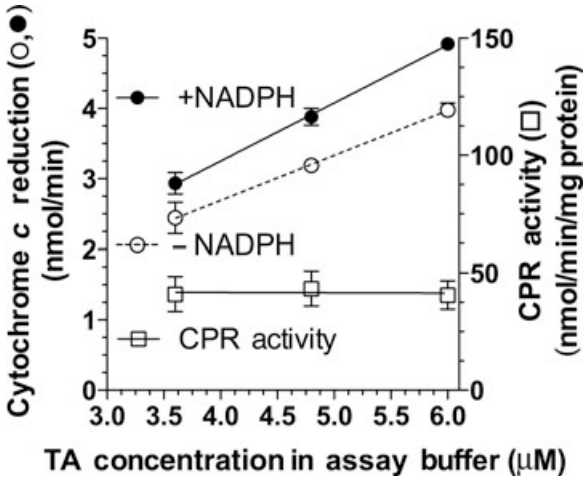

Figure 3. Cytochrome *c* reduction in the absence and presence of NADPH (left, *y*-axis) and CPR activity (right, *y*-axis) as a function of concentration of tannic acid (TA) in the assay buffer. Microsomal samples were treated with a single concentration (60 μM) of TA before determining their CPR activity using different volumes of the samples in the assay buffer. The symbols and bars represent the mean and SD values, respectively ($n = 3$).

cytochrome *c* reduction and TA concentrations in the range of 0.2–2.0 μM. Additionally, presence or absence of NADPH does not affect the reduction of cytochrome *c* by TA (Fig. 2).

Performance of the Modified Assay

On the basis of above results, a modified assay was designed, which utilizes the differences between the cytochrome *c* reduction activity of the sample in the absence and presence of NADPH (see *Materials and Methods*). To check the independence of the modified assay from the TA concentrations in the assay buffer, we kept the extent of inhibition of microsomal CPR activity constant by preincubating microsomes with a single 60-μM concentration of TA but used different volumes of TA-treated microsomes during the assay. This resulted in different concentrations of TA in the final assay buffer, causing different extent of direct cytochrome *c* reduction by TA. As demonstrated in Figure 3, an increase in the TA concentrations during the assay resulted in a linear increase in the absolute rates of cytochrome *c* reduction both in the presence and absence of NADPH. However, applying our method resulted in very similar ($p = 0.89$, one-way ANOVA) values for CPR activity for all three conditions (Fig. 3).

Inhibitory Effect of TA on Microsomal and Recombinant CPR Activity

The concentration–CPR inhibitory effect profiles of TA in RLMs and recombinant rat CPR are depicted in Figure 4. In RLMs (Fig. 4, top), TA showed a steep concentration–inhibitory response against CPR with IC_{50}, I_{max}, and Hill coefficient values (95% confidence intervals) of 58.2 (54.6–62.0) μM, 100% (89.1%–111%), and 6.47 (4.20–8.74), respectively. In the recombinant enzyme preparation (Fig. 4, bottom), the IC_{50}, I_{max}, and Hill coefficient values (95% confidence intervals) were 20.3 (14.2–29.0) μM, 99.9% (79.5%–120%), and 1.94 (0.789–3.10), respectively.

Inhibitory Effects of TA on the CPR Activity in Primary Rat Hepatocytes in the Presence of Serum

Figure 5 illustrates the time course and concentration dependency of the CPR inhibitory effect of TA in primary rat hepatocyte cultures in the presence of 10% FBS. Generally, the CPR inhibitory effect of a 250-μM concentration of TA increased with time, with a maximum of 50.4 ± 2.9% inhibition after 24 h (Fig. 5, top). The concentration dependency studies were then conducted after 24 h of incubation of TA with primary rat hepatocytes. This study showed concentration-dependent inhibition of CPR activity (Fig. 5, bottom) with IC_{50}, I_{max}, and Hill coefficient values (95% confidence intervals) of 275 (131–577) μM, 104% (58.5%–149%), and 1.40 (0.846–1.94), respectively.

Inhibitory Effects of TA on the CPR Activity in Primary Rat Hepatocytes in the Absence of Serum

The time course of the CPR inhibition by a single 100-μM concentration of TA and the concentration–response relationship of the TA effect in primary rat hepatocytes in KRH buffer in the absence of any protein in the medium are depicted in Figure 6. The inhibition of CPR by a single 100-μM concentration of TA in the medium significantly increased by an increase in time up to 8 h of incubation (Fig. 6, top). Additionally, the inhibition of CPR activity after 8 h of TA

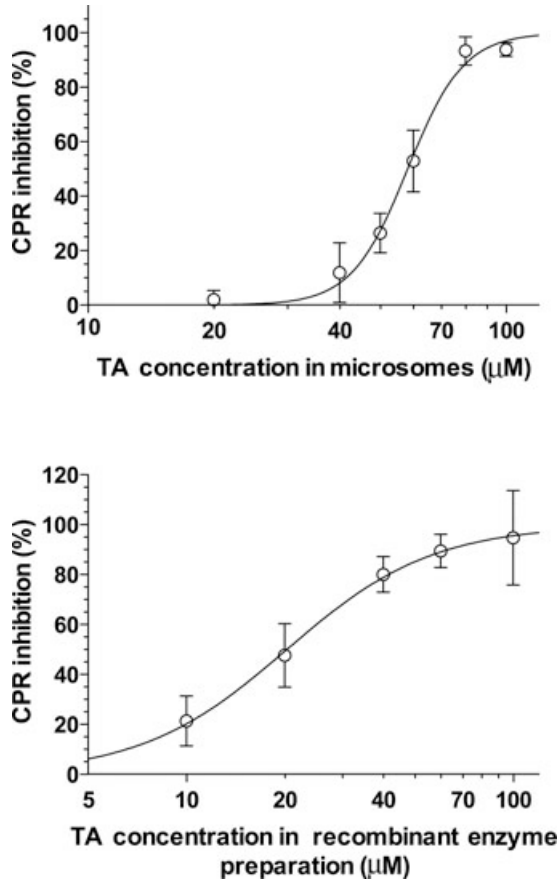

Figure 4. Concentration–CPR inhibitory effect profiles of tannic acid (TA) in rat liver microsomes (top) and recombinant CPR enzyme (bottom). The symbols and bars represent the mean and SD values, respectively ($n = 3$), and the line represents the best fit to the data.

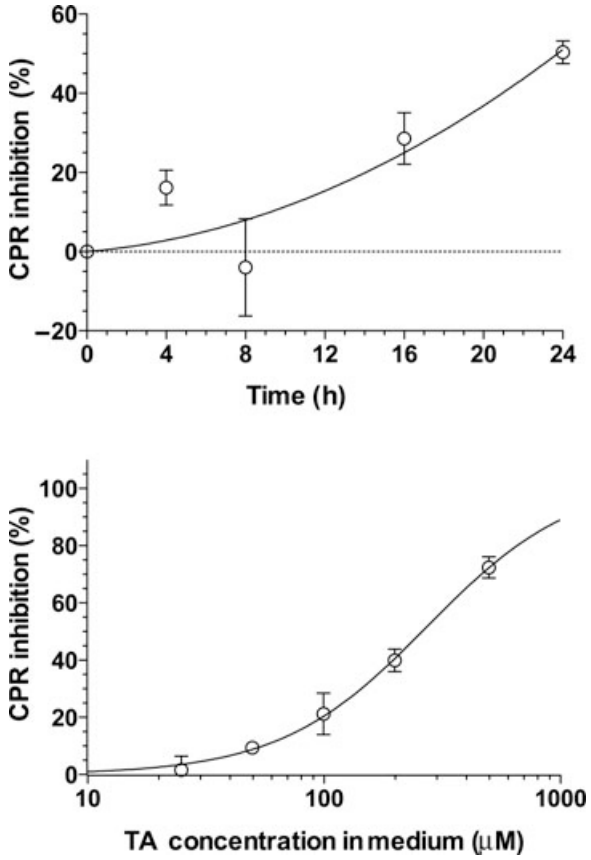

Figure 5. Time (top) and concentration (bottom) dependency of the inhibition of CPR activity by tannic acid (TA) in primary rat hepatocytes in the presence of serum-containing culture medium. The symbols and bars represent the mean and SD values, respectively ($n = 3$).

treatment showed a sigmoidal concentration dependency. Nonlinear regression analysis revealed IC_{50}, I_{max}, and Hill coefficient values (95% confidence intervals) of 54.6 (44.7–66.9) μM, 102% (93.9%–111%), and 2.12 (1.28–2.96), respectively.

Reversibility of TA-Mediated CPR Inhibitory Response in Primary Rat Hepatocytes

The degree of inhibition of CPR activity in a TA-free KRH buffer after 24 h of preincubation with a 250-μM concentration of TA in the culture medium is shown in Figure 7. Compared with baseline (time 0 immediately after 24 h of preincubation with TA), the CPR inhibitory activity of TA was retained by the hepatocytes during the 8 h of TA-free culture, with no significant differences between the 0- and 8-h activities (Fig. 7). However, after 4 h of TA-free incubation, the hepatocytes showed significantly ($p < 0.01$, one-way ANOVA, followed by Tukey's post-hoc test) higher degree of inhibition, compared with both the baseline and 8 h values (Fig. 7).

Effects of KCN on Microsomal and Hepatocytes CPR Activity

The effects of KCN (1 mM) in the assay buffer on the absolute values of CPR and the percentage inhibition of CPR activity by TA are presented in Table 1. Although KCN slightly decreased the absolute CPR activity of microsomes, it did not have any effect on the absolute values of CPR activity of hepatocytes. Notably, presence of KCN did not affect the extent of TA-mediated inhibition of CPR activity in either microsomes or hepatocytes (Table 1).

Effects of Inhibition of CPR by TA on Simulated IR Injury in Primary Rat Hepatocytes

Figure 8 demonstrates the effect of CPR inhibition by TA on an *in vitro* model of simulated IR injury in rat hepatocytes. Incubation of TA (100 μM) with rat hepatocytes 30 min before ischemia and during the ischemia (4 h) and reperfusion (20 min) period caused approximately 55% reduction ($p < 0.001$) in the CPR activity (Fig. 8a). However, the P450 inhibitor ABT did not show any inhibitory effect on the CPR activity

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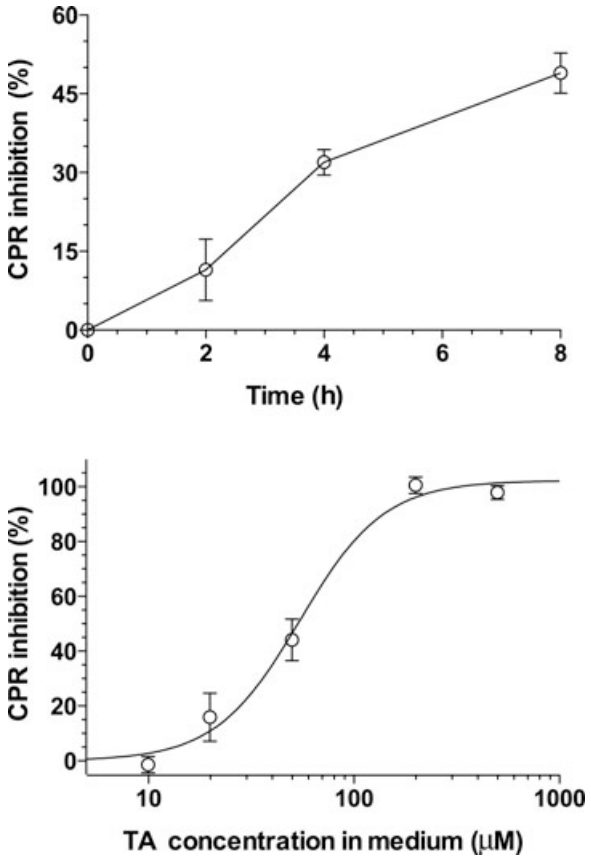

Figure 6. Time (top) and concentration (bottom) dependency of the inhibition of CPR activity by tannic acid (TA) in primary rat hepatocytes in the presence of serum-free buffer. The symbols and bars represent the mean and SD values, respectively ($n = 3$).

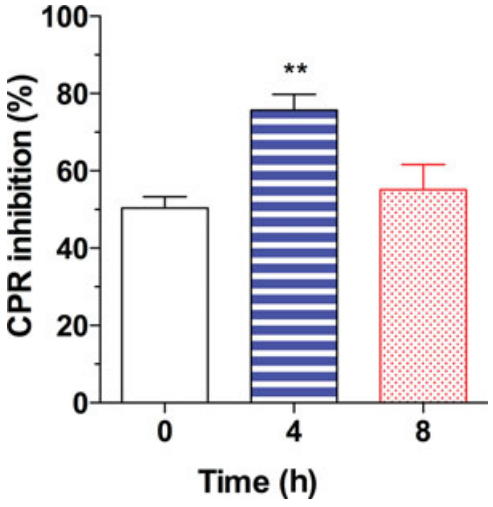

Figure 7. Reversal of tannic acid (TA)-mediated CPR inhibition in rat hepatocytes as a function of time in TA-free medium. Time 0 indicates 24 h of incubation in the presence of 250 μM TA in a serum-containing culture medium. The columns and bars represent the mean and SD values, respectively ($n = 3$). $^{**}p < 0.01$, compared with 0 or 8 h (ANOVA, followed by Tukey's post-hoc analysis).

(Fig. 8a). The decrease in the CPR activity by TA was associated with a significant ($p < 0.001$) decrease in ROS generation (by 59%) (Fig. 8b) and an increase in cell viability (from 30% to 52%) (Fig. 8c). As for ABT, a 37% reduction in ROS generation (Fig. 7b) was not associated with a significant improvement in cell viability (Fig. 8c).

DISCUSSION

Our laboratory has been interested in modulating the activity of CPR[6] to delineate its role in pathophysiological conditions associated with oxidative stress. A recent investigation[9] reported that TA is an inhibitor of CPR in rat and human liver microsomes. Therefore, we became interested in using TA in our studies. To our surprise, our initial investigations (Figs. 1 and 2) suggested that TA directly reduces cytochrome *c*, a substrate commonly used for CPR activity assay. Additionally, as opposed to the reduction of cytochrome *c* by CPR, which requires NADPH, this effect was NADPH independent (Fig. 2). An examination of the literature revealed that TA is capable of forming complexes with different proteins, including cytochrome *c*.[18,19] Additionally, more recently,[20] it was reported that TA forms a complex with Fe^{3+}, reducing it to Fe^{2+} in the following reactions:

$$\mathrm{TA} + n\mathrm{Fe}^{3+} \rightarrow (\mathrm{Fe}^{3+})_n - \mathrm{TA}$$
$$\rightarrow (\mathrm{Fe}^{2+})_n - \text{Oxidized TA}$$

Therefore, it is likely that the direct reduction of cytochrome *c* by TA, observed in our experiments (Fig. 2), is due to binding of TA to the Fe^{3+} moiety of cytochrome *c*, followed by reduction of Fe^{3+} to Fe^{2+}. Indeed, a previous study on the interactions of TA and proteins reported that addition of Tween 80 to a complex of TA–cytochrome *c* resulted in the release of cytochrome *c* with characteristic spectrum of the reduced form.[18]

The direct, NADPH-independent cytochrome *c* reducing effect of TA could potentially confound the true CPR activity of the sample in two opposing ways. First, the general procedure for the assay involves preincubation of cytochrome *c* and sample at 30°C [13] or 37°C[10] before the start of the reaction by the addition of NADPH. As demonstrated in Figure 1, in the presence of TA in the sample, this preincubation period would cause reduction of cytochrome *c* by TA, potentially leading to substrate (cytochrome *c*) depletion, thus reducing the rate of cytochrome *c* reduction after the addition of NADPH. In a second and opposing manner, presence of TA in the sample is expected to increase the rate of cytochrome *c* reduction in the presence of equal concentrations of cytochrome *c*. Therefore, depending on the length of the

Table 1. Effects of KCN in the Assay Buffer on the Absolute Values of CPR Activity and Percentage Inhibition of CPR Activity by Tannic Acid (TA, 50 μM) in Rat Liver Microsomes and Serum-Free Primary Hepatocytes

	Rat Liver Microsomes			Primary Rat Hepatocytes		
	nmol/min/mg		%	nmol/min/mg		%
	−TA	+TA	Inhibition	−TA	+TA	Inhibition
−KCN	130 ± 3	71.1 ± 9.9	45.3 ± 7.6	54.9 ± 10.7	24.1 ± 1.2	56.1 ± 2.2
+KCN	113 ± 4[a]	60.3 ± 2.4	46.6 ± 2.1	54.4 ± 3.4	21.2 ± 3.7	61.0 ± 6.8

[a] $p < 0.05$, two-way ANOVA with Bonferroni's post-test analysis.
Data are presented as mean ± SD ($n = 3$).

preincubation period (i.e., degree of substrate depletion), the presence of TA in the sample may potentially result in an increase, decrease, or no change in the apparent CPR activity of the sample using conventional cytochrome *c* reductase assays. Consequently, these artifactual effects are expected to confound the real CPR inhibitory effect of TA.

To circumvent the two problems associated with the presence of TA in the sample (substrate depletion and direct cytochrome *c* reduction), we modified the reported CPR assay[13] to accurately measure the true CPR inhibitory effect of TA. First, to prevent substrate depletion, we modified the assay by preincubating cytochrome *c* with NADPH and starting the assay by the addition of microsomes that contained TA. Additionally, we increased the concentration of cytochrome *c* to 100 μM (instead of 50 μM). Second, we utilized the different NADPH dependency properties of cytochrome *c* reduction by TA and CPR to correct the direct cytochrome *c* reduction by TA. To achieve this, we measured cytochrome *c* reduction activity of each sample twice, once in the absence (direct TA effect) and once in the presence (sum of CPR activity and direct TA effect) of NADPH and used the difference between the two measurements as the true CPR activity. Further studies (Fig. 3) showed that the estimated CPR activity using our modified method is independent of the concentration of TA in the assay mixture. These results suggest that our modified assay can accurately determine the CPR activity in the presence of TA in the assay buffer.

Using the modified method described above, we then measured the inhibitory potency of TA against rat liver microsomal CPR activity (Fig. 4, top). The IC_{50} of 58.2 μM, estimated from our studies in RLMs (Fig. 4, top), is substantially different from that previously reported (11.8 μM)[9] without consideration of TA-mediated direct cytochrome *c* reduction. Additionally, although TA showed complete inhibition of microsomal CPR activity at high concentrations in our RLM studies (I_{max} of 100%, Fig. 4, top), the I_{max} value in the previous report was around 80%.[9] Although the reported lower IC_{50} value could potentially be explained by a depletion of cytochrome *c* by TA during

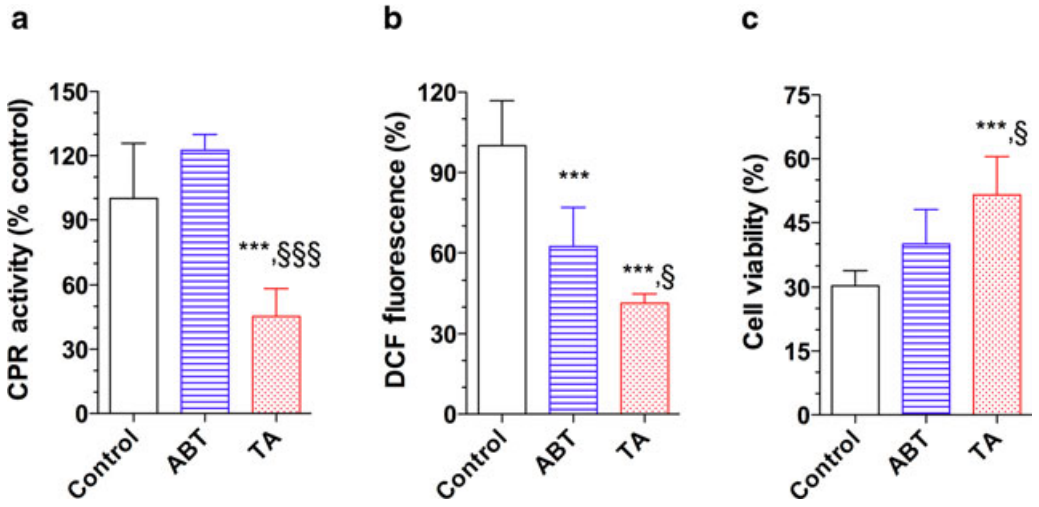

Figure 8. CPR activity (a), ROS generation (b), and cell viability (c) following simulated IR injury in untreated (control), tannic acid (TA)-treated (TA), and 1-aminobenzotriazole (ABT)-treated (ABT) primary rat hepatocytes. The columns and bars represent the mean and SD values, respectively ($n = 6$). $^{***}p < 0.001$, compared with control; $^{§}p < 0.05$ and $^{§§§}p < 0.001$, compared with ABT (ANOVA, followed by Tukey's post-hoc analysis).

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the preincubation period (Fig. 1), the lower reported I_{max} value could be attributed to a direct reduction of cytochrome *c* (Fig. 2) in the presence of complete inhibition of CPR at high TA concentrations. Nevertheless, the CPR inhibitory activity of TA reported here using our modified method (Fig. 4, top) is more likely to represent the true inhibitory activity of TA than that reported before[9] without consideration of the ability of TA to reduce cytochrome *c*.

The CPR inhibitory effect of TA in RLMs was further confirmed in a study with a recombinant preparation of rat CPR supersomes (Fig. 4, bottom). Because the CPR activity of the supersomes was approximately five times higher than that for RLMs (data not shown), we used approximately five times lower protein in our inhibitory experiments for the supersomes (2.25 μg supersomes vs 12 μg RLMs) to obtain comparable CPR activities. Therefore, the much lower IC_{50} value of 20.3 μM for the recombinant enzyme, compared with that for the RLMs (58.2 μM) (Fig. 4), is most likely due to a higher ratio of TA–sample protein for the recombinant enzyme.

Although TA was previously shown to inhibit CPR activity after its addition to liver microsomes,[9] there are no reports on the possible effects of TA on the CPR activity in primary hepatocytes or other liver cell lines. Primary hepatocytes are routinely used as an *in vitro* model of liver to investigate the xenobiotic mechanism of action and biotransformation.[21] Therefore, we extended our studies to evaluate the inhibitory potency of TA against CPR activity in primary rat hepatocytes.

In the presence of serum-containing culture medium, TA showed concentration- and time-dependent inhibition of CPR activity in primary rat hepatocytes (Fig. 5). The results of the time course study suggest that the uptake of TA (250 μM) into the hepatocytes in the presence of serum-containing medium is relatively slow, with maximum inhibitory response observed at the last incubation time of 24 h (Fig. 5, top). This is in contrast to the rapid (within 10 min) inhibition of CPR activity by TA in microsomes. Hence, we performed the concentration dependency studies after 24 h of incubation. Although not directly comparable, the IC_{50} value in primary rat hepatocytes (275 μM) was approximately 4.5-fold higher than that in microsomes (58.2 μM). Additionally, slope of the inhibitory effect–concentration relationship in primary rat hepatocytes (Fig. 5, bottom) was much shallower than that in the microsomes (Fig. 4, top) (Hill coefficients of 6.47 and 1.40, respectively). Overall, these data (Fig. 5) indicate that relatively high concentrations of TA and long incubation times are needed to substantially inhibit CPR activity in primary rat hepatocytes in serum-containing culture medium.

The relatively high IC_{50} value and delayed CPR inhibitory effects of TA in primary rat hepatocytes, observed in our studies (Fig. 5), is most likely due to a slow and inefficient uptake of TA by hepatocytes. Because the culture medium contained serum, we hypothesized that the high IC_{50} value and delayed effect might be due, at least in part, to binding of TA to the proteins in the medium, reducing the free concentration of TA available for cellular uptake. Indeed, our studies with primary rat hepatocytes in the absence of proteins (Fig. 6) showed a fivefold decrease in the IC_{50} value and faster inhibitory effect, compared with the data in the presence of proteins in the culture medium (Fig. 5). Interestingly, the IC_{50} value in the primary rat hepatocytes in the absence of proteins (54.6 μM) was very close to that in the liver microsomes (58.2 μM). These data are in agreement with previous reports,[22,23] which have shown that TA interacts with proteins, such as albumin, to form protein–TA complexes.

The results from our washout study showed that TA-mediated CPR inhibitory response was not reversible within 8 h of incubation of the hepatocytes with drug-free buffer (Fig. 7). Indeed, CPR inhibition following 4 h of drug-free incubation was even more than that at the baseline (Fig. 7). The lack of reversibility of CPR inhibition during the 8 h washout period suggests that the binding of TA to CPR is not easily reversible. This is in agreement with a recent report[24] indicating that TA causes irreversible inactivation of β-ketoacyl-ACP reductase, an enzyme involved in bacterial fatty acid synthesis. The reasons for an increase in the CPR inhibition at 4 h during the washout period (Fig. 7) are not immediately evident from our data. However, a slow and delayed intracellular trafficking of TA to the endoplasmic reticulum may be responsible for this delayed increase in the CPR inhibition in the absence of TA in the medium.

Various investigators have conducted the cytochrome *c*-based assay of CPR activity either in the presence or absence of KCN. The reasons for the inclusion of KCN are to inhibit the mitochondrial contaminant cytochrome *c* oxidase,[13,25] which may cause an underestimation of the CPR activity, and/or other reductases in the sample,[11] which may result in an overestimation of the activity. However, it has been reported that mitochondrial contamination of microsomal preparations and cell lysates from soft tissue, such as the liver, is low.[26] Additionally, the contributions of other reductases to the reduction of cytochrome *c* are reported to be minor for the mammalian samples, in contrast to that for the bacterial samples.[11] Nevertheless, to examine the effects of KCN on the outcome of our studies, we repeated our microsomal and hepatocyte experiments in the presence and absence of 50 μM TA and determined

the CPR activity of the samples both in the presence and absence of 1 mM KCN. In agreement with the literature data, KCN had little, if any, effect on the absolute values of CPR and no effect on our conclusions regarding the CPR inhibitory effect of TA (Table 1).

Our laboratory previously demonstrated that P450 induction[27] and inhibition[14,28] showed, respectively, exacerbation and attenuation of ROS generation and injury following warm hepatic IR in rats. Although CPR is known to generate ROS indirectly via P450 monooxygenase system[4] and directly via production of superoxide from molecular oxygen,[2,3] the role of CPR in oxidative stress and associated disorders remains unknown. Therefore, we used TA to study the effects of inhibition of CPR on ROS generation and cellular injury in an *in vitro* model of simulated IR in primary rat hepatocytes (Fig. 8). TA treatment caused significant reductions in the CPR activity (Fig. 8a) and ROS generation (Fig. 8b) and a significant increase in the cell viability (Fig. 8c) at 20 min after reperfusion. Although TA treatment also caused significant reductions in the CPR activity and ROS generation at 2 h after reperfusion, protection from cell death was absent at 2 h (data not shown). This may have been due to the reported nonspecific (adverse) effects of TA on rat hepatocytes[29] and liver,[30] which may have offset the decrease in the ROS generation at 2 h after reperfusion. Nevertheless, our data (Fig. 8) suggest that CPR may play a role in hepatic IR injury. However, because TA may exert other nonspecific effects, further studies using more specific inhibitors of CPR are needed to confirm the role of CPR in IR injury.

CONCLUSIONS

In conclusion, our studies showed that in addition to the previously reported CPR inhibitory activity in liver microsomes, TA directly reduces cytochrome *c*, which is commonly used for determination of CPR activity. Therefore, determination of CPR activity in the presence of TA may be incorrect if appropriate measures are not taken into account for the cytochrome *c*-reducing effects of TA. We have presented a modified assay to accurately measure CPR activity in the presence of TA. Furthermore, we have used the modified assay to characterize the concentration–effect relationship of TA for inhibition of CPR in RLMs, recombinant rat CPR, and primary rat hepatocytes. Finally, application of TA as an inhibitor of CPR is demonstrated in an *in vitro* model of IR in primary rat hepatocytes. These data may be useful in future studies using TA as an inhibitor of CPR in liver microsomes and primary hepatocytes.

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