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Redox Cycling Activity of Monoamine-Serotonin Binding Protein Conjugates

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ABSTRACT. It has been shown recently that the covalent binding of labelled dopamine and serotonin to serotonin binding proteins (SBP) from bovine frontal cortex is potently inhibited by their related neurotoxins. The present study reveals that the monoamine-SBP conjugates of serotonin, dopamine, and related toxins are able to catalyse redox cycling reactions. Using an improved method to detect quinoproteins in SDS-PAGE gels, we were also able to demonstrate that the redox cycling activity corresponded to two major protein components with molecular weights of 45 and 56 kDa. The covalent monoamine-SBP conjugates may be referred to as "artificial quinoproteins." BIOCHEM PHARMACOL 51;11:1521–1525, 1996.

KEY WORDS. serotonin binding proteins; neurotoxins; SBP-conjugates; redox cycling; artificial quinoproteins

Serotonin and catecholamines have recently been shown to bind to proteins from soluble extracts of calf brain in the presence of Fe^{2+} [1]. These proteins were referred to as SBP§ (45 and 56 kDa) [2, 3] and a model was proposed in which Fe^{2+} reacts with dissolved molecular oxygen to produce superoxide radicals that, in turn, oxidise monoamines into quinone derivatives (e.g. dopamine-o-quinone from dopamine, imine quinone from serotonin) that are prone to bind covalently to sulphydryl groups of proteins [4, 5]. Because the covalent binding of monoamines to SBP is increased by metals such as Mn^{2+} and Cu^{2+} [6] and potently inhibited by serotonin- and dopamine-related neurotoxins [7], it was proposed that this binding represents an *in vitro* model for cytotoxicity [4].

Although no universal view regarding the mechanism of monoamine neurotoxicity has emerged, three major theories have been proposed over the years: 1. Initial work by Saner and Thoenen [8] and subsequent studies by others [9–13] have led to the "alkylation theory." According to this theory, the oxidation products of dopamine- and serotonin-related toxins may undergo covalent attachment to proteins and, thereby, serve as an alkylating (cross-linking) agent of proteins. 2. The "free radical theory" suggests that the cytotoxic effect of monoamine neurotoxins is caused by highly reactive oxygen species: superoxide radicals ($^{\bullet}O_2$), hydrogen peroxide, and hydroxyl radicals ($^{\bullet}OH$). This theory is based on the fact that autoxidation of neurotoxins such as 6-hydroxydopamine and 5,6-dihydroxytryptamine is known to produce these oxygen species [12, 14] and that the hydroxyl radicals produce cell damage by peroxidation of membrane lipids and breaking of DNA [15]. 3. "Redox cycling" has been proposed as a possible mechanism to explain the neurodegenerative effects of 5,7-dihydroxytryptamine and many quinone derivatives [16, 17]. According to this mechanism, the monoamine should constantly cycle between its oxidised and reduced form, thereby depleting the cell's oxygen, as well as certain reducing agents such as NADPH.

The covalent monoamine-SBP conjugates may be referred to as "artificial quinoproteins." By definition, quinoproteins contain covalently or noncovalently bound quinonoid cofactors [18]. Several natural quinoproteins have been found in bacteria, as well as in mammalian cells with enzymatic functions [19]. They contain cofactors, such as pyrroloquinoline quinone, 6-hydroxydopa quinone (topa quinone), and tryptophan tryptophylquinone, and their redox cycling properties (i.e. reduction of the quinone to quinol and reoxidation to quinone) have been used for qualitative or quantitative estimation [20, 21]. A more sensitive test was developed by Paz *et al.* (1991) and Glatz *et al.* (1995) wherein, at pH 10, glycine is used as a reducing agent and Nitroblue tetrazolium (NBT) as the oxidising agent. The production of formazan, the reduced form of NBT, can then be measured both spectrophotometrically

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§ *Abbreviations:* SBP, serotonin binding proteins; 5-HT, serotonin; DA, dopamine; 6-OHDA, 6-hydroxydopamine; 5,6-DHT, 5,6 dihydroxytryptamine; 5,7-DHT, 5,7 dihydroxytryptamine; 6,7-diOHTIQ, 6,7 dihydroxy,1,2,3,4 tetrahydroisoquinoline; $Na_2S_2O_5$, sodium metasulfite.

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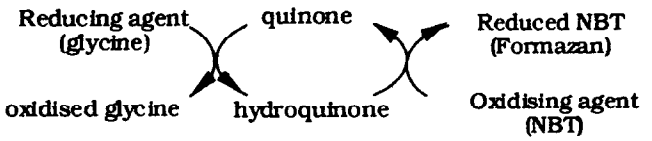
and electrophoretically. The reactions can be described as follows:



We report here that some of the covalent monoamine-SBP conjugates are good redox cycling catalysts as measured by the reduction of NBT. These results may help to clarify the biological significance of monoamine-SBP conjugates. It is suggested that the redox cycling activity of conjugates, such as the free tryptamine and quinone derivatives, could disturb the cell's metabolism in a long-lasting fashion because of the cell's difficulty in eliminating the immobilised monoamine.

MATERIALS AND METHODS

Materials

Dopamine hydrochloride, 5-HT (serotonin), 5,6-DHT, 5,7-DHT, 6-hydroxydopamine hydrobromide, adrenochrome, and nitroblue tetrazolium (NBT) were from Sigma (St. Louis, MO, U.S.A.); iron sulphate ($FeSO_4$) and dithiothreitol (DDT) from Aldrich Chemie (Belgium); 6,7-dihydroxy-TIQ were from Janssen Chimica (Belgium); and $Na_2S_2O_5$ was from U.C.B. (Belgium) and glycine from Merck (Germany).

Preparation of Covalent Monoamine-SBP Conjugates

Calf brains were obtained from a local slaughterhouse and kept on ice during transportation. All manipulations were performed at 0–4°C. Soluble SBP-containing extracts were prepared as previously described [1]. Protein concentrations were determined according to Lowry *et al.* [22] and bovine serum albumin was used as standard. Samples of extract (1 mg protein/mL in 20 mM potassium phosphate buffer at pH 7.5) were incubated for 15 min at 20°C with 1 mM serotonin, dopamine, or related neurotoxins along with 100 μM freshly prepared $FeSO_4$ or 1 mM sodium metabisulfite (as a negative control). $FeSO_4$ was added immediately after the monoamines. After the incubation, 0.3 mL of the mixture was applied to a small sephadex G-50 column (0.7 × 15 cm) equilibrated with phosphate buffer and eluted with the same buffer to separate the SBP-monoamine conjugates from free monoamine by molecular sieving. The void volume (1.5 mL) was discarded and the fraction containing monoamine-SBP conjugates (1.8 mL) was collected.

Redox Cycling Assay

SBP-quinoproteins were detected by Nitroblue tetrazolium (NBT) reduction [20]. Briefly, 500 μL column eluated monoamine-SBP conjugate (25 μg protein) was added to 1 mL of 2 M potassium glycinate buffer (pH 10), and the reaction was started by addition of 1 mL of 0.25 mM NBT in potassium glycinate buffer. Formazan production (ϵ = 15000 M^{-1} cm^{-1}) was measured spectrophotometrically at 530 nm within intervals of 5 min up to 30 min.

Polyacrylamide Gel Electrophoresis

Protein extract (1 mg protein/mL) was incubated for 15 min at 20°C in 20 mM potassium phosphate buffer (pH 7.5) with 1 mM serotonin, dopamine, or related neurotoxins along with 100 μM freshly prepared $FeSO_4$ or 1 mM sodium metabisulfite. After this incubation, a 25-μL sample of each mixture was prepared under reducing conditions (i.e. diluted with buffer containing 50 mM dithiothreitol) and heated for 30 sec at 100°C. SDS polyacrylamide electrophoresis (SDS-PAGE 10%) gels were performed as described by Laemmli [23]. Gels (20 μg protein/slot) were stained for proteins by Coomassie Brilliant Blue R 250 or for redox cycling activity in a reaction mixture containing 0.25 mM NBT in 2 M potassium glycinate buffer (pH 10) for 1–3 hr in the dark according to Glatz *et al.* [21].

RESULTS AND DISCUSSION

The potential of SBP-conjugated dopamine, serotonin, and related neurotoxins to catalyse "redox cycling" phenomena was assessed by using the NBT/glycine system. This system, applied earlier to detect the redox cycling activity of natural quinoproteins [20], is based on the ability of glycine to act as the reducing agent and the ability of NBT to act as terminal oxidation agent. Catalysis of this reduction-oxidation reaction promotes the conversion of NBT into formazan, a product that can be detected spectrophotometrically (ϵ = 15000 M^{-1} cm^{-1}).

Several of the SBP-monoamine conjugates promoted the formation of formazan. As shown in Fig. 1A,B, the rate of this reaction (nM formazan formed per min) remained steady for up to 30 min, so that it could be calculated by linear regression of the absorbance vs time plot. The potency of SBP-monoamine conjugates to trigger formazan formation decreased in the following order: SBP-adrenochrome > SBP-6-OH-dopamine ≈ SBP-5,6DHT > SBP-6,7-diOH-TIQ ≈ SBP-dopamine > SBP-5HT ≈ SBP-5,7DHT (Table 1). SBP was unable, by itself, to catalyse formazan formation; thus, indicating that the effect was related to the presence of monoamine. Reducing agents such as sodium metabisulfite are able to prevent monoamine oxidation, and we have previously shown that they effectively prevent their covalent coupling to SBP [4]. When sodium metabisulfite was present in the initial reaction step in which dopamine was added to SBP, the resulting protein fraction also was unable to catalyse the formation of formazan (Table 1). This suggests that the catalytic activity of the SBP-monoamine conjugates was mediated by covalently bound monoamine, rather than by remnants of free monoamine. This view was further supported by use of the SDS-PAGE-based method developed by Glatz *et al.* [21] for the direct detection of quinoproteins. Electrophoresis patterns of the SBP-conjugates are depicted in Fig. 2A.

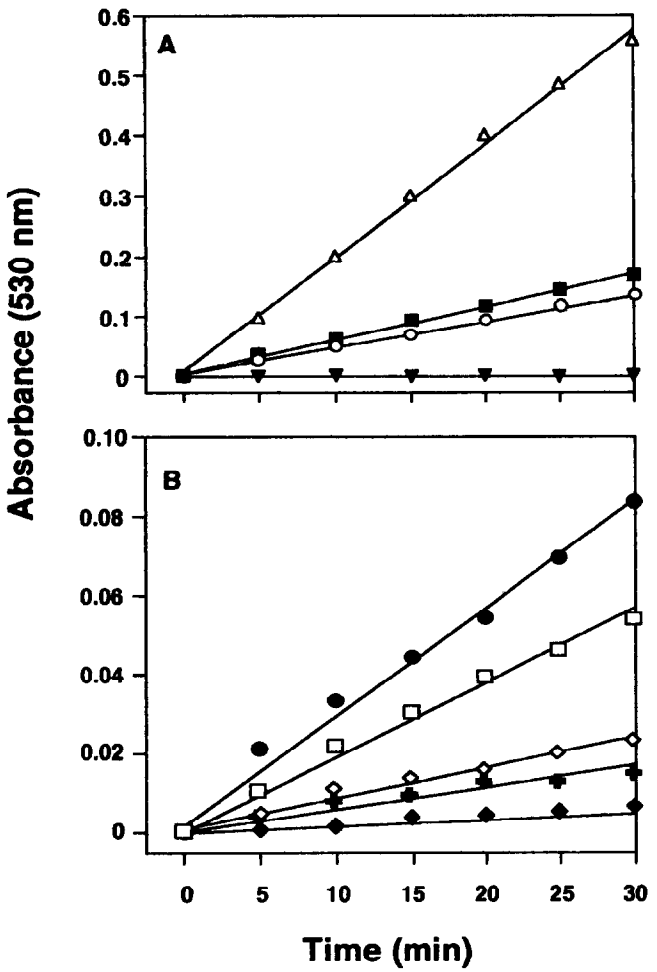


FIG. 1. Redox cycling activity of monoamine-SBP conjugates. Samples of monoamine-SBP conjugates were prepared as described in the Methods section. Formazan production was measured spectrophotometrically at 530 nm within intervals of 5 min up to 30 min. Absorbance values are means of 3 experiments. Panel A: SBP-adrenochrome (△), SBP-6-hydroxydopamine (■), SBP-5,6DHT (○). Panel B: SBP-6,7 diOH-TIQ (●), SBP-dopamine (□), SBP-5,7DHT (◇), SBP-serotonin (+). As negative controls, SBP were treated either with Fe^{2+} alone (▼) or with 1 mM dopamine in the presence of 1 mM sodium metabisulfite (◆). Kinetics of formazan formation are given in Table 1.

Redox cycling reactivity (corresponding to formazan formation) was clearly visible in two bands. In agreement with the spectrophotometric determinations, staining was most pronounced for the SBP-adrenochrome, SBP-6-OH-dopamine, and SBP-5,6DHT conjugates. Comparison of these gels with those stained for proteins (Coomassie Brilliant Blue R 250) (Fig. 2B) revealed that the redox cycling activity corresponded to proteins with molecular weights of 45 and 56 kDa. In addition, proteins with comparable molecular weights were previously shown to be the main targets for binding of labelled dopamine and serotonin [1]; in fact, they can be shown to represent the major components of SBP-containing brain extracts (Fig. 2B). This is in full

TABLE 1. First-order rate constants for the redox cycling activity of monoamine-SBP conjugates

Conjugate	Formazan production (nM/min)
SBP-Adrenochrome	6330 ± 317
SBP-60HDA	2000 ± 120
SBP-5,6 DHT	1665 ± 80
SBP-6,7-diOHTIQ	1000 ± 30
SBP-DA	665 ± 18
SBP-5,7 DHT	330 ± 10
SBP-5HT	330 ± 15
SBP + $Na_2S_2O_5$ + DA	0
SBP + Fe^{2+} alone	0

The experimental values are shown in Fig. 1. The optical densities at 530 nm were converted in terms of formazan concentration (ϵ + 15000 M^{-1} cm^{-1}) and the concentration vs time plots were subjected to linear regression analysis to yield the formazan production in nM per min. Values are means ± SD of 3 independent experiments.

agreement with our earlier suggestion that oxidised monoamines may covalently bind to proteins without discrimination, provided they bear accessible cysteine residues [4].

The disparity in redox cycling catalytic potency of the various SBP conjugates, as determined both by the spectrophotometric and electrophoretic approaches, could be due to inborn differences between the physicochemical properties of the conjugated monoamines, as well as to differences in the amount of bound monoamine. In this context, it has been shown that the binding of 6-OH-dopamine to proteins is much more pronounced than for dopamine [24]. Poor binding of 5,7DHT may also explain the low redox cycling activity of SBP-5,7DHT conjugates, despite the presumption that redox cycling plays a major role in the cytotoxicity of free 5,7DHT [16].

Conjugation of adrenochrome and 5,6DHT with SBP occur simultaneously with the formation of high-molecular-weight protein aggregates. These can be observed at the beginning of the separating gel both by protein staining and by their ability to catalyse redox cycling (Fig. 2A and B, lane 2 and 10). A similar phenomenon has been shown to occur when intact liver cells are treated with menadione, a 1,4-napthoquinone-containing drug [25]. Because such aggregates are absent when brain extracts are incubated with Fe^{2+} alone (Fig. 2A, lane 3), it is likely that adrenochrome and 5,6DHT play an active role in the aggregation process. One possibility is that they could serve as alkylating, cross-linking agents for proteins. This property has been reported for 5,6DHT quinone [11]. Alternatively, monoamines could produce free oxygen radicals during autoxidation and thereby produce oxidative cross-linking between proteins. Such a mechanism is probably of lesser importance because 6-OH-dopamine, a monoamine that is well known for its ability to produce free oxygen radicals during autoxidation [26], did not induce appreciable amounts of high-molecular-weight aggregates (Fig. 2A, lane 4). Moreover, the free oxygen radicals generated by the reaction of Fe^{2+} with molecular oxygen were also ineffective under our experimental conditions.

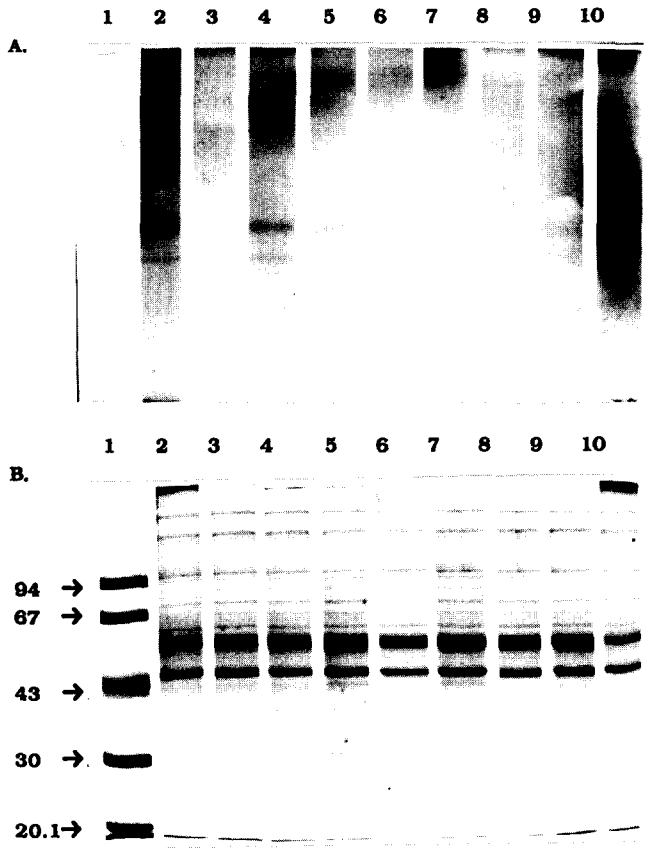


FIG. 2. SDS-PAGE of monoamine-SBP conjugates. Samples of monoamine-SBP conjugates were prepared as described in the Methods section and subjected to SDS-PAGE. A. Gels were treated with 0.25 mM NBT in 2 M potassium glycinate buffer (pH 10) to detect redox cycling activity. B. Proteins were stained with Coomassie Brilliant Blue R 250. Molecular weight markers (Lane 1) were: phosphorylase b, 94 kDa; bovine serum albumine, 67 kDa; ovalbumine, 43 kDa; carbonic anhydrase, 30 kDa; soybean trypsin inhibitor, 20.1 kDa. As control, SBP were treated with 100 μM Fe^{2+} alone (lane 3) or with 1 mM dopamine in the presence of 1 mM sodium metabisulfite (lane 6). SBP were treated with 100 μM Fe^{2+} in the presence of 1 mM of the following monoamines: adrenochrome (lane 2), 6-hydroxydopamine (lane 4), dopamine (lane 5), 6,7 diOH-TIQ (lane 7), serotonin (lane 8), 5,7DHT (lane 9), and 5,6DHT (lane 10).

Catecholamines and serotonin have been reported to form covalent bonds with intracellular proteins in intact cells [27–29]. When present in a proper environment, such as in the active site of enzymes, bound monoamines could play a constructive physiological role. For example, it has been established that 6-hydroxydopa/6-hydroxydopaquinone, the quinoid component of bovine serum amine oxidase, plays a functional role at the active site of this enzyme [30]. However, uncontrolled conjugation of oxidised monoamines to proteins could impair the cell's physiological functions. First, the activity of certain enzymes (e.g. catechol-O-methyltransferase) or proteins (e.g. G proteins) have been shown to be altered by the bound monoamine [31, 32]. Second, as has been shown for liver cells on treatment with menadione [25], the conjugation process could happen parallel to the formation of protein aggregates. Third, the present study reveals that the conjugates are able to catalyse "redox cycling" of the NBT/glycine system. These conjugates may therefore be regarded as "artificial quinoproteins," and it is likely that they produce cell damage by redox cycling. Indeed, quinone toxicity has been associated with redox cycling in many studies [17] and, because of the covalent nature of the monoamine-SBP conjugates, they should escape clearance from the cell so that their catalytic effect could outlast that of the free monoamines. In this view, the present findings comply with our suggestion [4] that the binding test initially developed by Tamir and coworkers [2, 3] to show the presence of "serotonin binding proteins" is more likely to constitute an *in vitro* model for neurotoxicity.

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