Biochemical Pharmacology, Vol. 56, pp. 1121–1128, 1998.

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00136-1

Characterization of the Enzymatic Activity for Biphasic Competition by Guanoxabenz (1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine) at α_2-Adrenoceptors

II. DESCRIPTION OF A XANTHINE-DEPENDENT ENZYMATIC ACTIVITY IN SPLEEN CYTOSOL

Maija Dambrova, Staffan Uhlén,* Christopher J. Welch,† Peteris Prusis* and Jarl E. S. Wikberg*‡*

*Department of Pharmaceutical Biosciences, Division of Pharmacology and †Department of Pharmaceutical Chemistry, Division of Organic Chemistry, Uppsala University, Uppsala, Sweden

ABSTRACT. The mechanism for formation of high affinity binding of guanoxabenz (1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine) to α_2-adrenoceptors by the rat spleen cytosol was studied. We report here that the spleen cytosolic fraction mediated the reduction of guanoxabenz to guanabenz(1-(2,6-dichlorobenzylidene-amino)-3-guanidine), the latter having an almost 100-fold higher affinity for rat α_{2A}-adrenoceptors than guanoxabenz itself. The reaction product could be separated by high-performance liquid chromatography and its identity as guanabenz confirmed by nuclear magnetic resonance. The spleen cytosolic activity could be separated into high and low molecular weight components, the high molecular weight component requiring low molecular weight factors for maximal activity. Xanthine oxidase seems to be the most likely candidate responsible for the activity, as the guanoxabenz-reducing activity of the high molecular weight component could be sustained by exogenously applied xanthine, while it was potently blocked by allopurinol. The conversion of guanoxabenz by the cytosolic activity was also quite potently blocked by DWO1, 1-(3,4-dimethoxybenzylideneamino)3-hydroxyguanidine, a hydroxyguanidine analogue to guanoxabenz. BIOCHEM PHARMACOL **56**;9:1121–1128, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *N*-hydroxyguanidine, guanoxabenz; metabolic conversion; reduction; spleen; xanthine oxidase

In earlier studies, we showed that the *N*-hydroxyguanidine guanoxabenz§ appears to bind to α_2-adrenoceptors in different tissues with both low and high affinities [1–3]. For example, guanoxabenz appeared to bind with markedly higher apparent affinity to α_2-adrenoceptors in spleen membranes compared to cerebral cortex α_2-adrenoceptors [2]. Moreover, we found evidence that the high affinity guanoxabenz binding was due to a metabolic activation of guanoxabenz, leading to the formation of a product that showed high affinity for the α_2-adrenoceptors, which gave rise in some tissues and under some conditions to biphasic competition curves that could be resolved into two apparent affinities of guanoxabenz for α_2-adrenoceptors [4]. In addition, in an accompanying paper to the present one, we have recently shown that the spleen membrane basal enzymatic activity responsible for the activation of guanoxabenz could be inhibited by allopurinol, as well as by a series of *N*-hydroxyguanidine analogues of guanoxabenz. Moreover, we demonstrated that the enzymatic activity was inhibited by extensive washing of the spleen membranes and that the activity in the washed membranes could be partially restored by the addition of xanthine [5].

In the present study, we have further characterized the activity that seemingly promotes conversion of guanoxabenz to a metabolite that shows *ca.* 100-fold higher affinity for α_2-adrenoceptors than guanoxabenz itself. Our present results show that the major activity in the spleen is localized to the cell cytosol, and that this cytosolic activity can be inhibited by an *N*-hydroxyguanidine analogue of guanoxabenz, in a similar manner as in spleen membranes. We have also found that cytosolic guanoxabenz-reducing activity can be activated by xanthine and blocked by allopurinol. Moreover, we show herein that the addition of graded doses of spleen cytosol to cerebral cortex membranes induces biphasicity of guanoxabenz competition curves in α_2-adrenoceptor assays using cerebral cortex membranes,

‡ Corresponding author: Dr. Jarl E. S. Wikberg, Pharmaceutical Pharmacology, BMC, Box 591, SE-751 24 Uppsala, Sweden. Tel. +46-18-4714238; FAX +46-18-559718; E-mail: Jarl.Wikberg@farmbio.uu.se

§ *Abbreviations:* DMF-d7, *N*,*N*-dimethylformamide-d7; DWO1, DWO1, 1-(3,4-dimethoxybenzylideneamino)3-hydroxy-guanidine; Guanabenz, 1-(2,6-dichlorobenzylidene-amino)-3-guanidine; Guanoxabenz, 1-(2,6-dichloro benzylidene-amino)-3-hydroxyguanidine; [^{3}H]RX821002, (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline.

Received 26 September 1997; accepted 11 March 1998.

which gives strong support to the notion that the previously observed biphasic competition curves of guanoxabenz result from the metabolic activation of the compound. Our results are discussed in terms of the possibility that cytosolic guanoxabenz-converting activity resides in xanthine oxidase.

MATERIALS AND METHODS

Preparation of Tissue Membrane and Cytosolic Fractions

Male Sprague–Dawley rats weighing 200–300 g were decapitated and the spleens and cerebral cortices rapidly excised and homogenized in 1:10 v/w ice-cold 50 mM Tris-HCl, 5 mM EDTA, 0.1 mM PMSF (phenyl methyl sulphonyl fluoride), 10 μg/mL soybean trypsin inhibitor and 200 μg/mL of bacitracin, pH 7.5, using a motor-driven teflon glass homogenizer. The homogenates were spun at 500 × *g* for 15 min thereafter the supernatants were collected. Aliquots of the thus formed crude supernatants were frozen and stored at −80° for later use. The remaining crude supernatants were spun at 38,000 × *g* for 30 min. The second supernatants were also frozen and stored at −80° for later use for the studies of the cytosolic enzyme activity. (The second supernatant will subsequently be referred to as the "spleen cytosolic fraction" whereas the first supernatant, described above, will be referred to as the "crude spleen preparation"). The membraneous pellets from the 38,000 × *g* centrifugation of the spleen and cerebral cortex preparations were twice resuspended and recentrifuged in 1.5 mM EDTA, 50 mM Tris-Cl pH 7.5. Final pellets were diluted to protein concentrations of *ca*. 2.4 mg protein/mL with 1.5 mM EDTA, 50 mM Tris-Cl pH 7.5. Aliquots of the membranes were frozen and stored at −80° until use. Protein was determined according to Lowry *et al.* [6].

Sephadex g-50 Chromatography

0.5 mL of rat spleen cytosolic fraction were loaded onto a 10 mL of G-50 Sephadex® column that had been preequilibrated with 1.5 mM EDTA, 50 mM Tris-HCl, pH 7.5 buffer. The column was then eluted with the same buffer and 1 mL fractions collected. All the chromatographic procedures were carried out at 4°.

Assay of the Guanoxabenz-Converting Activities

Guanoxabenz-converting activities were assayed by using various α_2-adrenoceptor binding protocols. Activation of guanoxabenz can be observed as an increase in its apparent affinity, nonactivated guanoxabenz having a K_i of *ca*. 4000 nM and the fully activated form *ca*. 40 nM for an α_{2A}-adrenoceptor. In routine assays, α_{2A}-adrenoceptor-containing membranes from rat spleen or cerebral cortex were incubated with the α_{2A}-adrenoceptor-selective radioligand [^{3}H]RX821002 (approximately 1 nM) and competing drugs, as indicated, in 150 μL of 33 mM Tris-Cl, pH 7.5, 1 mM EDTA, 140 mM NaCl, 2 mM $MgCl_2$, 100 μM of Gp-p(NH)p (guanyl-5′-yl-imido-diphosphate) for 1 hr at 25° [7]. In some experiments, spleen cytosol or various Sephadex® G50 gel filtration chromatographic fractions were first incubated with 6 μM of guanoxabenz for 1 hr at 25°, thereafter the reaction mixture was heated to 85° for 5 min and centrifuged to remove protein precipitates. Aliquots of the supernatants (1:6 final dilution, 1 μM of guanoxabenz in final binding assay) were then assayed using the above α_2-adrenoceptor binding protocol. Termination of the binding assays were performed by filtering and washing on Whatman GF/C filters. All assays were performed in duplicate. Data were analyzed using a radioligand binding analysis package (Wan System), essentially as described [8–10].

HPLC Separation of Spleen Cytosol Guanoxabenz Reaction Product

To obtain enough of the product for NMR studies, guanoxabenz (500 μM) was preincubated with the spleen cytosolic fraction for 1 hr at 25°C. In these incubations, the 25 mM Tris-HCl, 0.75 mM EDTA medium was supplemented with $MgCl_2$ (2 mM) and dithiothreitol (2 mM) because the additions were found to enhance the enzymatic activity. Total volume of the reaction mixture was 4 mL. After the incubation, the reaction mixture was heated in a water bath to 80° for 5 min. The sample was then centrifuged and the supernatant filtered through a 0.22 μM Millipore syringe filter, thereafter 200 μL of the filtrate were injected onto a reverse phase chromatography column (Waters Symmetry™ C8) equilibrated in 40% methanol, 60% 10 mM Na-acetate buffer, pH 4.5. The elution speed was 0.5 mL/min, and the eluted substances were detected by a UV monitor set at 260 nM (2151 LKB variable Wavelength UV monitor). The fractions corresponding to the product peak were lyophilized, dissolved in DMF-d7 and used for NMR analysis.

NMR Recordings

NMR spectra were recorded at 270.2 MHz over the range 0.0–10.0 ppm in DMF-d7 using a JEOL JNM 270 spectrometer equipped with a standard 5 mM probe and, for the microsample observation, a 5-mm probe with inverse configuration. Samples of guanoxabenz and guanabenz were dissolved in 0.6 mL of DMF-d7 to give a concentration of approximately 0.1 M. Spectra were recorded using 64 K data points and 16 transients. The sample of enzymatic origin was dissolved in 20 μL DMF-d7 and drown into an open capillary tube. The tube was sealed using rubber plugs and suspended coaxially in a 5-mm NMR tube. The ^{1}H spectrum was obtained using 64 K data points and 2048 transients. Data were processed using the DELTA processing package.

Isotopes, Drugs, and Chemicals

[^{3}H]RX821002 (51 Ci/mmol) was from Amersham, UK. Guanoxabenz was from Rousell, Romainville, France. DWO1 had been synthesized as described by Doubell *et al.* [11]. Dicumarol, menadione, allpurinol, xanthine and guanabenz were from Sigma. Sephadex® G-50 was from Pharmacia Biotech.

RESULTS

Demonstration of Guanoxabenz-converting Activity in Spleen Cytosol

In order to characterize the activity responsible for the conversion of guanoxabenz to a high affinity α_2-adrenoceptor binding metabolite, we probed the eventual cytosolic localization of the activity in the spleen. In these experiments, the α_2-adrenoceptor binding activity of guanoxabenz was assessed by using cerebral cortex membranes and the radioligand [^{3}H]RX821002. The cerebral cortex was selected because it contains predominantly α_{2A}-adrenoceptors and because we have previously demonstrated that cerebral cortex membranes do not contain any basal guanoxabenz-converting activity [2]. The results are shown in Fig. 1A. As can be seen from the figure, the presence of spleen cytosol in the binding assay (1:3 final dilution) led to an approximate 100-fold leftward shift of the guanoxabenz competition curve compared to the control, where no spleen cytosol had been added (K_i = 4600 ± 1600 nM in the absence of cytosol versus K_i = 39 ± 20 nM in the presence of cytosol; mean ± SEM, n = 2). As a control, guanabenz competition curves were obtained using a cerebral cortex membrane spleen cytosol mixture (1:3 final dilution of spleen cytosolic fraction) (Fig. 1A); the K_i of guanabenz was found to be 28 ± 3 nM, close to the K_i values that we found earlier for guanabenz using the plain rat spleen and cerebral cortex membranes [2]. When the spleen cytosolic fraction had been heat inactivated to 85° for 5 min, its addition to the cerebral cortex membranes did not lead to any increase in the apparent binding affinity of guanoxabenz (K_i = 3700 ± 800 nM). Moreover, Fig. 1A presents the competition curve of guanoxabenz using the crude spleen preparation and [^{3}H]RX821002. (In this case, the α_2-adrenoceptors used for binding are residing in the spleen membranes). As can be seen from Fig. 1A, the guanoxabenz competition curve was in this case superimposed over the cerebral cortex membrane curve where spleen cytosol had been added, as well as being superimposed over the guanabenz control curve. For the crude spleen preparation, the apparent K_i of guanoxabenz was 43 ± 5 nM. Thus, these results indicate that the spleen cytosol added to the cerebral cortex brought about the same degree of activation of the guanoxabenz as did the crude spleen preparation. Moreover, the K_i of the activated guanoxabenz aligns well with the K_i of guanabenz.

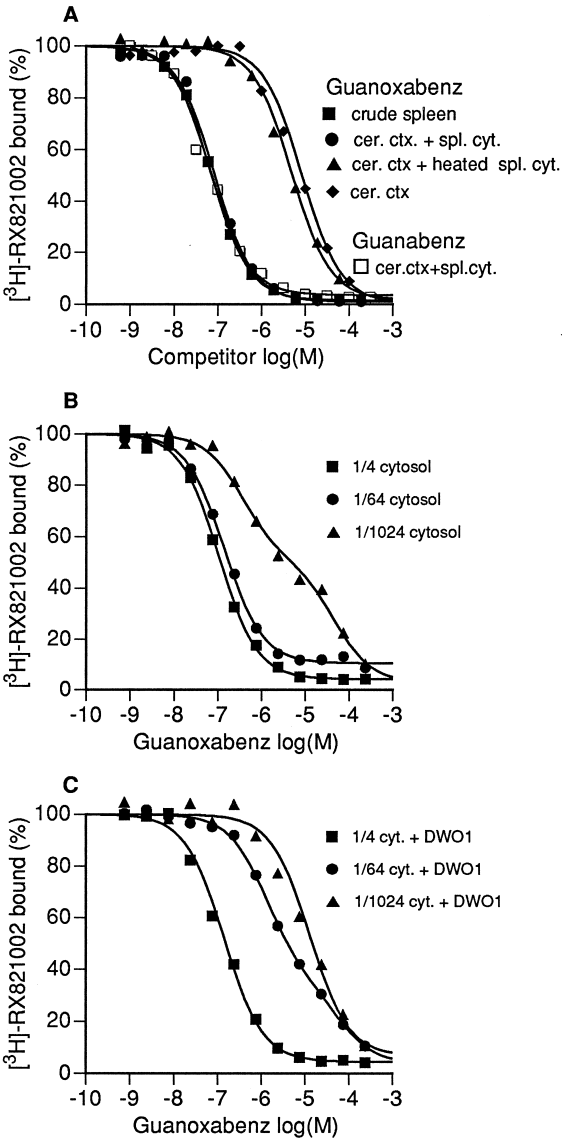

FIG. 1. Results from experiments performed with rat cerebral cortex and spleen membranes (containing α_2-adrenoceptors), complemented with spleen cytosol (containing guanoxabenz-converting enzyme). Panel A shows curves for the competition of guanoxabenz with the binding of [^{3}H]RX821002 to α_{2A}-adrenoceptors in the crude spleen preparation (■) (see Methods for details), cerebral cortex membranes (◆), cerebral cortex membranes in the presence of three-fold diluted spleen cytosol (●), and in cerebral cortex membranes in the presence of heat-inactivated (85° for 5 min) three-fold diluted spleen cytosol (▲). A control competition curve of guanabenz using cerebral cortex membranes in the presence of three-fold diluted spleen cytosol is also shown (□). Panels B and C show competition of guanoxabenz with the binding of [^{3}H]RX821002 to α_{2A}-adrenoceptors in cerebral cortex membranes in the presence of spleen cytosol. In C, various dilutions of spleen cytosol had been preincubated with 6 μM of DWO1 for 40 min before addition to the α_{2A}-adrenoceptors binding assay (1.5 μM of DWO1 in final assay). The experiment in B shows the corresponding control where the spleen cytosol was incubated for 40 min in the absence of DWO1. The cytosol incubations were diluted four-fold in the final binding assay, the final dilutions of the cytosol preparation becoming 4- (■), 64- (●), and 1024-fold (▲), respectively.

Demonstration of the Ability of the N-Hydroxyguanidine DWO1 to Inhibit the Guanoxabenz-converting Activity in Spleen Cytosol

In previous studies, we showed that the guanoxabenz-converting activity of spleen membranes could be inhibited by a series of *N*-hydroxy-guanidine analogues of guanoxabenz [4, 5]. In the present study, we wanted to test if the cytosolic activity was also inhibited by such an *N*-hydroxy-guanidine. In these studies, we selected the *N*-hydroxy-guanidine DWO1 [11], having found it to be one of the most potent compounds in inhibiting the guanoxabenz-converting activity in spleen membranes. In order to assess an inhibitory activity of DWO1 in the spleen cytosol, we preincubated different dilutions of the spleen cytosolic fraction with or without 6 μM DWO1 for 40 min at 25°. The preincubated cytosol was then mixed with cerebral cortex membranes and various concentrations of guanoxabenz added together with 1 nM [^{3}H]RX821002. The incubations were continued for a further 60 min, and the amount of [^{3}H]RX821002 bound to the cerebral cortex α_{2A}-adrenoceptors determined. After the last four-fold dilution into the assays, the final dilutions of the spleen cytosolic fractions in these experiments were 4-, 64- or 1024-fold. The results from the control experiments (i.e. without DWO1) are shown in Fig. 1B, and the results from the DWO1-treated cytosols performed with the same membranes and on the same occasion are shown in Fig. 1C. For the 4- and 64-fold diluted cytosolic fractions of the controls, an essentially complete activation of guanoxabenz leading to high affinity binding took place, the K_i values being 63 ± 11 nM and 110 ± 20 nM, respectively (mean ± SEM, n = 2, Fig. 1B) (these results are to be compared with those of Fig. 1A, where the activated guanoxabenz gave a K_i of *ca*. 40 nM). However, when the cytosol was diluted 1024-fold, only a partial activation of guanoxabenz was observed, as is evident from the biphasic appearance and the shift of the competition curve to the right (K_i-high = 290 ± 40 nM and K_i-low = 37,000 ± 23,000 nM; Fig. 1B). By contrast, when the cytosol had been pretreated with 6 μM of DWO1, a clear shift of the guanoxabenz competition curve was already evident at a 64-fold dilution of the cytosolic fraction. For the four-fold dilution, only one K_i-high value of 85 ± 9 nM was observed, whereas for the 64-fold dilution at K_i-high and a K_i-low of 290 ± 40 nM and 42,000 ± 21,000 nM, respectively, were seen. For the 1024-fold dilution, only a K_i low of 6500 ± 2100 nM was observed (mean ± SEM, n = 2; Fig. 1C). In addition, we showed in subsequent experiments that the addition of 6 μM of DWO1 did not affect the competition curves of guanabenz in the cerebral cortex membranes that had been supplemented with the spleen cytosolic fraction (data not shown). These data thus show that the ability of DWO1 to modify apparent α_2-adrenoceptor affinities is not a general phenomenon shared by other α_2-adrenoceptor active compounds besides guanoxabenz. Taken together, our data seem to indicate that DWO1 inhibits the guanoxabenz-converting activity of the spleen cytosol in a similar fashion as it does the guanoxabenz-converting activity of the spleen membranes as we had demonstrated previously.

Evidence for Macromolecular Nature of Spleen Cytosolic Guanoxabenz-converting Activity

In order to investigate whether the guanoxabenz-converting activity of the spleen cytosol resided on a macromolecule, we added spleen cytosolic fractions (0.5 mL) to 10 mL of Sephadex G-50 columns and eluted them stepwise in 1 mL portions. The void was eluted in fraction No. 1 and contained essentially all high molecular weight materials, whereas low molecular weight materials were eluted in fractions No. 6–15. The guanoxabenz-converting activities of the eluted fractions were assayed by preincubating them with 6 μM of guanoxabenz (1:3 dilution of the fractions), followed by heat inactivation of the reaction mixtures, centrifugation to remove precipitated protein, and the addition of an aliquot of the resulting supernatant (final dilution 1:6, resulting in 1 μM of guanoxabenz in binding assay) to a radioligand binding assay that contained appropriate concentrations of [^{3}H]RX821002 and cerebral cortex membranes (see Methods for details). Results are shown in Fig. 2A. As can be seen from the figure, only fraction No. 1 (the void) contained weak guanoxabenz-converting activity, giving a *ca*. 30% decrease in [^{3}H]RX821002 binding. It was hypothesized that the relatively low activity of this fraction was due to the loss of a low molecular weight co-factor needed for activity of a macromolecular entity. Therefore, fraction No. 1 was reconstituted with fractions No. 2–15, and then preincubated with 6 μM of guanoxabenz, thereafter the mixtures were tested in the same assay as described above. As can be seen from Fig. 2A, the addition of fractions No. 7–14 to fraction No. 1 potentiated the guanoxabenz-converting activity markedly. The highest potentiating activity appeared in fraction No. 9, where the [^{3}H]RX821002 binding was reduced by an additional ~50% as compared to the reduction induced by fraction No. 1 alone. In other experiments, we found that heating fractions No. 2–15 to 85° for 5 min did not affect their ability to potentiate the activity of fraction 1 (data not shown). Experiments were repeated 2–3 times, giving essentially identical results. Altogether, these data suggest that guanoxabenz-converting activity in the spleen cytosol consists of a high molecular weight component (presumably an enzyme), and that this component requires heat-stable molecular weight co-factor(s) for its guanoxabenz-converting activity.

Effect of Xanthine and Allopurinol on the Guanoxabenz-converting Activity of Desalted Spleen Cytosol

Experiments were performed to evaluate the effect of xanthine and allopurinol on the activity of the Sephadex G-50 fraction No. 1, which was obtained as described in the preceding paragraph. Fraction No. 1 (1:6 final dilution) was

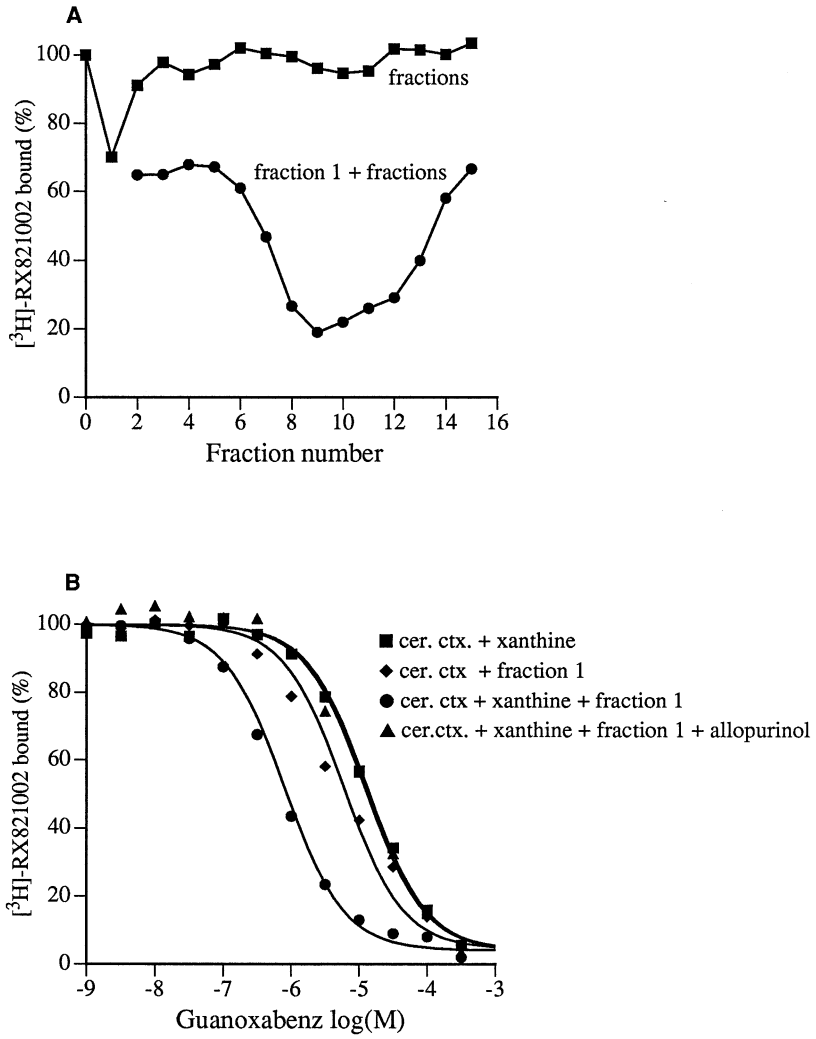

FIG. 2. Assays of guanoxabenz-converting activity of G-50 Sephadex gel filtration fractions using cerebral cortex membranes and [^{3}H]RX821002 binding. Panel A shows the effect of incubating the chromatographic fractions with 1 μM of guanoxabenz on the binding of [^{3}H]RX821002 to cerebral cortex α_{2A}-adrenoceptors. Squares (■) represent assays of fractions No. 1–16 alone (1:18 final dilution). Bullets (●) represent assays of fraction No. 1 (1:18 final dilution) in combination with fractions No. 2–16 (1:18 final dilution). (The 100% level in panel A represents the binding of [^{3}H]RX821002 in the presence of 1 μM guanoxabenz.) Panel B shows the effects of the combinations of fraction No. 1, xanthine and allopurinol on the the ability of guanoxabenz to compete with [^{3}H]RX821002 binding to α_{2A}-adrenoceptors in membranes from the cerebral cortex. The concentration of xanthine used was 100 μM and that of allopurinol 20 μM. For the assays containing G-50 fraction No. 1, the dilution of the fraction was nine-fold. For controls not containing the G-50 fraction, a corresponding amount of elution buffer was added.

incubated with 1 nM [^{3}H]RX821002, cerebral cortex membranes, and varying concentrations of guanoxabenz in the presence and absence of 100 μM of xanthine and/or 20 μM allopurinol for one hour, whereafter the amount of [^{3}H]RX821002 bound to the cerebral cortex α_{2A}-adrenoceptors was assayed. The resulting competition curves are shown in Fig. 2B. As can be seen from the figure, the guanoxabenz competition curve obtained with the addition of fraction No. 1 was located slightly to the left (K_i = 3300 ± 1400 nM) of the curve obtained in the presence of xanthine only (K_i = 6300 ± 700 nM). When both xanthine and fraction No. 1 were present, the guanoxabenz curve was shifted far to the left (K_i = 180 ± 60 nM). When 20 μM of allopurinol was also added in the presence of xanthine and fraction No. 1, the competition curve was shifted back to the right, so that it was superimposed on the curve obtained in the presence of xanthine only, the K_i value of guanoxabenz becoming 4200 ± 800 nM (mean ± SEM, n = 2). In separate sets of control experiments, we showed that 100 μM xanthine and/or 20 μM of allopurinol did not affect guanabenz competition curves in the cerebral cortex (data not shown).

Characterization of the Enzymatic Reaction Product by NMR

To obtain enough of the product for NMR studies, guanoxabenz (500 μM) was preincubated with the spleen cytosolic fraction for 1 hr at 25° and then analyzed by HPLC, as described in the Methods section. In separate experiments, it was shown that our chromatographic set-up clearly separated guanoxabenz and guanabenz; the retention times were *ca.* 10.5 and 12 min, respectively (Fig. 3, top) Pilot experiments showed that the product peak increased in size with incubation time (from 5 min to 1 h), while the peak corresponding to guanoxabenz concomitantly decreased in size. Addition of guanabenz to the incubation mixture increased the area of the product peak, without distorting its symmetry (data not shown). After 1 h, the peak corresponding to guanabenz represented more than 95% of the UV-absorbing material eluted from the column at 10–15 min (Fig. 3, bottom). The fractions containing the product peak were collected, pooled and lyophilized, and subjected to NMR analysis. The NMR spectrum obtained is shown in Fig. 4 together with the

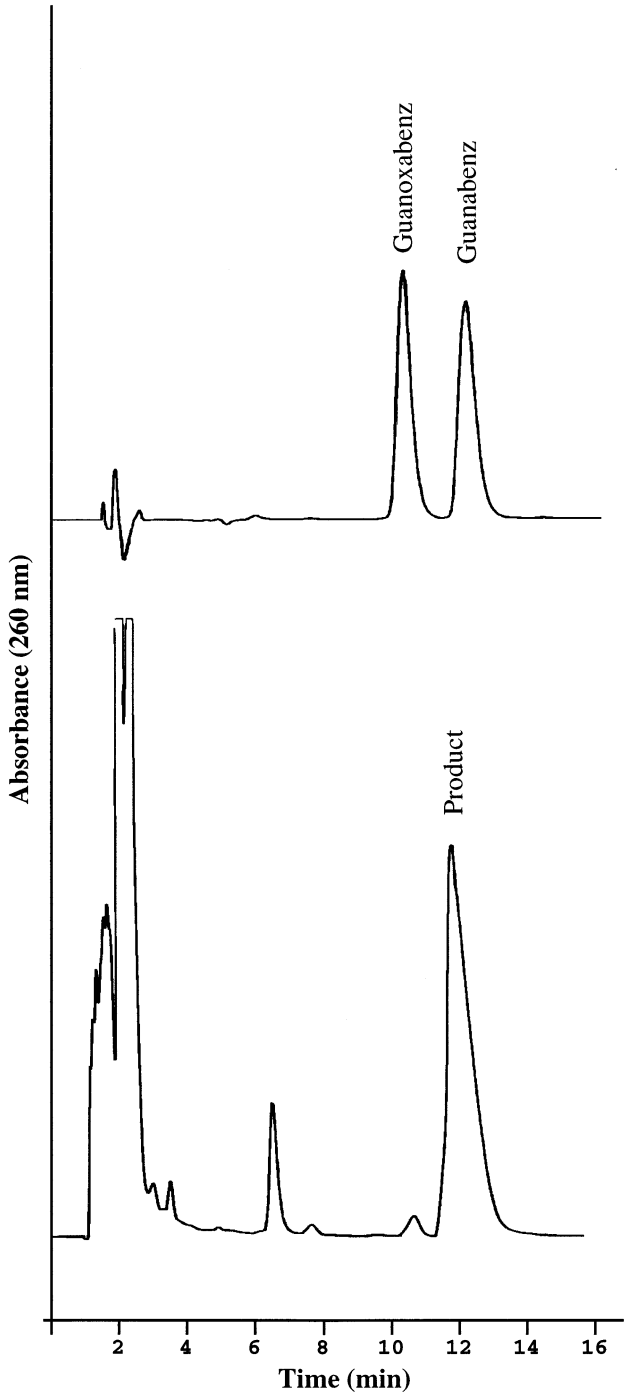

FIG. 3. HPLC of a mixture of 20 μM of guanoxabenz and 20 μM of guanoxabenz mixture (top) and a representative chromatogram of the enzymatic reaction mixture of guanoxabenz and spleen cytosol (bottom) (see text for details). Column: Waters Symmetry C8. Mobile phase: 40% methanol, 60% 10 mM Na-acetate buffer, pH 4.5. Flow rate 0.5 mL/min. Detection: 260 nm.

spectra for native guanoxabenz and guanabenz. As can be seen, the spectrum of the enzymatic reaction product showed a sharp singlet at 8.51 ppm, with its spectrum exactly corresponding to that of guanabenz.

DISCUSSION

In the present study, we have further characterized the enzymatic activities responsible for the increased affinity of guanoxabenz for α_2-adrenoceptors. The results of the present study reinforce our previous interpretation that the apparent bimodal high and low affinity binding of guanoxabenz to α_2-adrenoceptors is due to an enzymatic activation of guanoxabenz (Fig. 5). The data of the present paper speaking in favour of this interpretation are as follows.

First, the addition of spleen cytosol to cerebral cortex membranes (the latter not containing any capacity of their own to bind guanoxabenz with the higher affinity) led to an almost 100-fold increase in the apparent affinity of guanoxabenz for the cerebral cortex α_{2A}-adrenoceptors. As the ability of the spleen cytosol to convey this activation of guanoxabenz was destroyed upon heat inactivation of the spleen cytosol, the most straightforward explanation for this finding is that the cytosol contains an enzymatic activity that provides the activation.

Second, the spleen cytosol could be separated into its high and low molecular weight components, the presence of both being required for maximal activity (Fig. 2A). Moreover, the results of Fig. 2B show that the activity of the desalted high molecular weight component could be sustained by the addition of xanthine, and that allopurinol, which is known to be a potent inhibitor of xanthine oxidase [12, 13], could completely block the effect induced

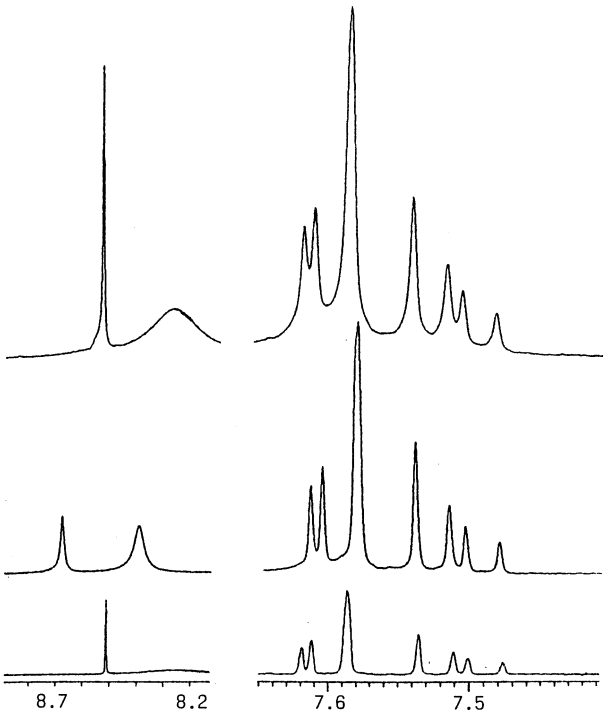

FIG. 4. Two hundred and seventy MHz ^{1}H-NMR spectra (8.9 to 8.1 ppm and 7.65 to 7.45 ppm regions) for 95 μM of guanabenz (top), 90 μM of guanoxabenz (middle) and approximately 100 μM product obtained from enzymatic treatment of guanoxabenz (bottom). All substances were dissolved in DMF-d7.

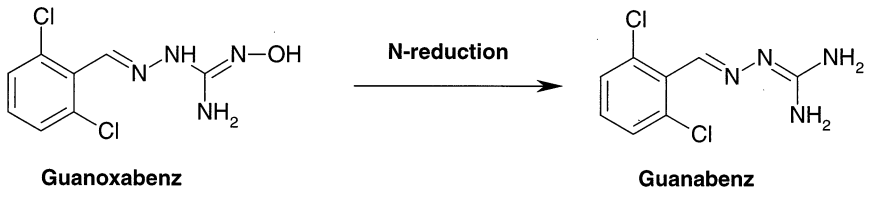

FIG. 5. Proposed enzymatic N-reduction of guanoxabenz.

by xanthine. These results thus make xanthine oxidase a strong candidate for the enzyme responsible for the conversion of guanoxabenz to the high affinity metabolite.

Thirdly, we have shown herein that spleen cytosol catalyzes the conversion of guanoxabenz to a product whose chromatographic properties and NMR spectrum are identical to those of guanabenz. These results thus provide definite evidence that the high affinity metabolite of guanoxabenz is in fact guanabenz. Guanabenz is known to be an α_2-adrenoceptor agonist, and we have previously measured its affinity for α_{2A}-adrenoceptors in the rat cerebral cortex and rat spleen by using [^{3}H]RX821002 radioligand binding, the K_i of guanabenz being *ca.* 18–27 nM [2]. The affinity of the activated guanoxabenz measured in the present paper (K_i = 39 ± 20 nM; Fig. 1A) aligns well with the K_i of guanabenz for the α_{2A}-adrenoceptor, again in support of the hypothesis.

In our earlier studies [1–3, 5], we used membranes mainly prepared from the spleen and to some extent also from kidney, and showed that guanoxabenz could apparently bind with both high and low affinities to the α_2-adrenoceptors present in these membranes. We presume that the activity of the spleen (and kidney) membranes resides in the same enzyme as that of the spleen cytosol. The activity in the membranes could be due to traces of the soluble enzyme that was not washed away during the preparation of the membranes. It is quite clear that the activity in the membranes is far weaker than that of the spleen cytosol. In fact, we presume that the previously observed biphasic competition curves of guanoxabenz, which are resolvable into two site fits by using radioligand binding computer modeling analysis, arise due to a rate-limited conversion of guanoxabenz to guanabenz. By applying the law of mass action, it can easily be shown that a mixture of two compounds that show unequal affinities for one site will give rise to uniphasic competition curves that resolve only into one site fit. However, if the compound with the lower affinity is metabolically converted to one with the higher affinity, and the rate of formation of the high affinity compound becomes limited at the higher concentration range, then the competition curves would become shallow and be resolvable into two site fits. (In fact, we have recently performed an extensive mathematical evaluation for the conditions generating biphasic competition curves when a competitor is subjected to metabolic transformation [14], and we refer the reader to this study for further discussion on the subject). In the present study, spleen cytosol, even diluted as much as 1:64 (Fig. 1B), was capable of effecting essentially complete activation of guanoxabenz. As is also evident from Fig. 1B, further dilution of the spleen cytosol leads to biphasic competition curves, which is completely in line with the models mentioned above [14]. We presume that at high dilution the converting capacity of the soluble enzyme would become limited, either due to a limited amount of enzymatic capacity or by the depletion of endogenous co-factor(s).

In two of our earlier papers [4, 5] we showed that a series of hydroxyguanidines were capable of seemingly inhibiting the conversion of guanoxabenz to its high affinity metabolite in the rat spleen membranes. In the present study, we have shown that one of the most potent hydroxyguanidines in this respect, DWO1, is also capable of inhibiting the guanoxabenz-converting activity of the spleen cytosol (Fig. 1C). The exact mechanism by which DWO1 affects the inhibition of guanoxabenz-converting activity is at present unknown and merits further study.

Several series of *N*-hydroxyguanidines have been synthesized during the last decade and reported to have antiviral and anticancer activities [15–17], but little is known about their exact metabolic fate *in vivo*. Hydroxyguanidine itself was found to be reduced to guanidine when injected intraperitonealy into rats, or when it was incubated with rat or guinea pig liver membrane homogenates [18]. In earlier studies, Clement and coworkers have reported the *N*-reduction of *N*-hydroxydebrisoquine *in vitro* [19] as well as the retroreduction of guanoxabenz to guanabenz by a microsomal NADH-dependent system [20]. The same group recently showed that the microsomal *N*-reduction of guanoxabenz is catalyzed by an enzyme system composed of cytochrome B_5, NADH cytochrome B_5-reductase, and benzamidoxime reductase [21]. It thus seems plausible that guanoxabenz can become metabolically reduced by several distinct processes in the body one of which might be xanthine oxidase, as indicated by the present study, as well as by cytochromes, as indicated by the studies of Clement *et al.*

In summary, we have demonstrated herein that the spleen cytosol is capable of reducing guanoxabenz to guanabenz. The activity requires endogenous co-factor(s) present in the spleen cytosol, or exogeneously applied xanthine, for its activity. As the metabolic conversion of guanoxabenz can be blocked by allopurinol, we have discussed the results in terms of possibility that the activity is mediated by xanthine oxidase.

This work was supported by the Swedish MRC (04X-05957) and the Wiberg foundation. Staffan Uhlén received supported of the Berth von Kantzow foundation.

References

1. Uhlén S and Wikberg JES, Delineation of three pharmacological subtypes of α_2-adrenoceptors in the kidney. *Br J Pharmacol* **104:** 657–664, 1991.
2. Uhlén S, Xia Y, Chhajlani V, Lien EJ and Wikberg JES, Evidence for the existence of two forms of α_2A-adrenoceptors in the rat. *Naunyn–Schmiedebergs Arch Pharmacol* **347:** 280–288, 1993.
3. Xia Y, Uhlén S, Chhajlani V, Lien EJ and Wikberg JES, Further evidence for the existence of two forms of α_2B-adrenoceptors in the rat. *Pharmacol Toxicol* **72:** 40–49, 1993.
4. Wikberg JES, Chhajlani V, Xia Y, Muceniece R and Uhlén S, Newer subtypes of the α_2? In: *Adrenoceptors* (Ed. RR Ruffolo), pp. 109–118. Harwood Academic Publishers, Luxembourg, 1995.
5. Uhlén S, Dambrova M. Tiger G, Oliver DW and Wikberg JES, Characterization of the enzymatic activity for biphasic competition by guanoxabenz (1-(2,6-dichlorobenzylideneamino)-3-hydroxyguanidine) at α_2-adrenoceptors. I. Description of an enzymatic activity in spleen membranes. *Biochem Pharmacol* **56:** 1111–1119, 1998.
6. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
7. Uhlén S, Xia Y, Chhajlani V, Felder CC and Wikberg JES, [^{3}H]-MK 912 binding delineates two α_2-adrenoceptor subtypes in rat CNS one of which is identical with the cloned pA2d α_2-adrenoceptor. *Br J Pharmacol* **106:** 986–995, 1992.
8. De Lean A, Hancock AM and Lefkowitz RJ, Validation and statistical analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol Pharm* **21:** 5–16, 1982.
9. Bergström A and Wikberg JES, Guanine nucleotides regulate both agonist and antagonist binding to cod brain α_2-adrenoceptors. *Acta Pharmacol Toxicol* **59:** 270–278, 1986.
10. Uhlén S and Wikberg JES, Delineation of rat kidney α_2A- and α_2B-adrenoceptors with [^{3}H]RX821002 radioligand binding: Computer modeling reveals that guanfacine is an α_2A-selective compound. *Eur J Pharmacol* **202:** 235–243, 1991.
11. Doubell PCJ and Oliver DW, Synthesis and anti-HIV-1 activity of *N*-hydroxy-N^1-aminoguanidines. *Drug Res* **42:** 65–69, 1992.
12. Massey V, Komai H and Palmer G, On the mechanism of inactivation of xanthine oxidase by allopurinol and other pyrazolo[3,4-d]pyrimidines. *J Biol Chem* **245:** 11, 2837–2844, 1970.
13. Hille R and Massey V, Tight binding inhibitors of xanthine oxidase. *Pharmac Ther* **14:** 249–263, 1981.
14. Wikberg JES, Dambrova M, Uhlén S and Prusis P, Conditions for biphasic competition curves in radioligand binding for ligands subjected to metabolic transformation. *Biochem Pharmacol* **56:** 1129–1137, 1998.
15. Hui MB, Lien EJ and Trousdale MD, Inhibition of human adenoviruses by 1-(2′-hydroxy-5′-methoxybenzylidene) amino-3-hydroxyguanidine tosylate. *Antiviral Res* **24:** 261–73, 1994.
16. Lori F, Malykh A, Cara A, Sun D, Weinstein JN, Lisziewicz J and Gallo RC, Hydroxyurea as an inhibitor of human immunodeficiency virus-type 1 replication. *Science (Washington DC)* **266:** 801–805, 1994.
17. Chern J–W, Leu Y–L, Wang S–S, Jou R, Lee C–F, Tsou P–C, Hsu S–C, Liaw Y–C and Lin H–M, Synthesis and cytotoxic evaluation of substituted sulfonyl-*N*-hydroxyguanidine derivatives as potential antitumor agents. *J Med Chem* **40:** 2276–2286, 1997.
18. Walker JB and Walker MS, The enzymatic reduction of hydroxyguanidine. *J Biol Chem* **234:** 1481–1484, 1959.
19. Clement B, Schultze–Mosgau M–H and Wohlers H, Cytochrome P450 dependent *N*-hydroxylation of guanidine (debrisoquine), microsomal catalysed reduction and further oxidation of the *N*-hydroxy-guanidine metabolite. *Biochem Pharmacol* **46:** 2249–2267, 1993.
20. Clement B, Demesmaeker M and Line S, Microsomal catalyzed *N*-hydroxylation of guanabenz and reduction of the *N*-hydroxylate metabolite: Characterization of the two reactions and genotoxic potential of guanoxabenz. *Chem Res Toxicol* **9:** 682–688, 1996.
21. Clement B and Demesmaeker M, Formation of guaoxbenz from guanabenz in human liver. A new metabolic marker for CYP1A2. *Drug Metab Disp* **25:** 1266–1271, 1997.