Possible Role of Salsolinol Quinone Methide in the Decrease of RCSN-3 Cell Survival

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The endogenous dopamine-derived neurotoxin salsolinol was found to decrease survival in the dopaminergic neuronal cell line RCSN-3, derived from adult rat substantia nigra in a concentration-dependent manner (208 μM salsolinol induced a 50% survival decrease). Incubation of RCSN-3 cells with 100 μM dicoumarol and salsolinol significantly decreased cell survival by 2.5-fold ($P < 0.001$), contrasting with a negligible effect on RCHT cells, which exhibited nearly a 5-fold lower nomifensine-insensitive dopamine uptake. The levels of catalase and glutathione peroxidase mRNA were decreased when RCSN-3 cells were treated with 100 μM salsolinol alone or in the presence of 100 μM dicoumarol. *In vitro* oxidation of salsolinol to *o*-quinone catalyzed by lactoperoxidase gave the quinone methide and 1,2-dihydro-1-methyl-6,7-isoquinoline diol as final products of salsolinol oxidation as determined by NMR analysis. Evidence of the formation of salsolinol *o*-semiquinone radical has been provided by ESR studies during one-electron oxidation of salsolinol catalyzed by lactoperoxidase.

Key Words: **salsolinol; quinone methide; dopamine; DAT; DT-diaphorase; lactoperoxidase; dicoumarol; catalase; glutathione peroxidase; substantia nigra.**

Dopamine-derived isoquinolines, i.e., salsolinol (SAL, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline) and its methylated derivative(s), have been suggested to act as endogenous dopaminergic neurotoxins, inducing selective neuronal cell death and eliciting symptoms almost identical to idiopathic Parkinson's disease in humans (1). However, only *N*-methyl-(*R*)-salsolinol has been reported to induce behavioral changes similar to those observed in Parkinson's disease (2). (*R*)-1,2(*N*)-Dimethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline and its oxidation product 1,2(*N*)-dimethyl-6,7-dihydroxyisoquinolinium ion accumulate in the nigrostriatal system in the human brain (3). It has been hypothesized (1) that metabolic bioactivation (nonenzymatic oxidation or catalyzed by semicarbazide-sensitive oxidase) of SAL and/or its methylated derivatives to isoquinolinium ions, and their intracellular accumulation (3) through selective uptake by the dopamine transporter system, are the key mechanisms underlying the neurotoxicity of isoquinolines. This specific oxidative pathway can induce inhibition of the mitochondrial respiratory chain, thereby triggering ATP depletion and an energy crisis (4).

The interactions of SAL with oxidative enzymes (tyrosinase, ceruloplasmin and peroxidase) have also been reported (5). The well-established oxidative degradation of SAL resulted in the formation of an unstable electrophilic *o*-quinone and its tautomeric product (5). Recently, the formation of SAL-melanin, its spectroscopic characteristics and redox transfer properties were demonstrated as the final products of salsolinol oxidative degradation (6). The possibility of an intervention of peroxidative enzymes in the melanization process involving isoquinoline occurring in neural cells under pathological conditions is, thus, quite plausible (6).

The question to be addressed is whether SAL acts as a toxic molecular product by itself, or if it is oxidatively metabolized *in situ* to highly reactive species. Thus, the possible role of oxidative metabolism in salsolinol neurotoxicity has been investigated in the present work

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using a dopaminergic cell line derived from adult rat substantia nigra (RCSN-3) (7, 8). The role of dicoumarol (DIC), a specific inhibitor of DT-diaphorase has also been investigated (9). The regulation of antioxidant enzymes during the treatment of RCSN-3 cells with SAL in the absence or presence of dicoumarol was studied by using RT-PCR. The oxidative pathways of salsolinol were studied *in vitro* by using lactoperoxidase (LPO) and NMR and ESR analysis for the identification of the SAL oxidation products.

EXPERIMENTAL PROCEDURES

Chemicals. All chemicals and lactoperoxidase (LPO) [EC 1.11.1.7) were purchased from the Sigma Chemical Co. (St. Louis, MO) with the exception of H_2O_2, which was purchased from AnalaR BDH (Darmstadt, Germany).

Incubations conditions. The concentrations of LPO were measured spectrophotometrically using appropriate molecular extinction coefficient (10).

Cell culture. The RCSN-3 cell line was derived from the substantia nigra (7, 8). The RCSN-3 cell line grows on monolayers, with a doubling time of 52 h, a plating efficiency of 21% and a saturation density of 410,000 cells/cm^2. The cultures were kept in an incubator at 37°C with 100% humidity and an atmosphere of 10% CO_2. The cells were grown in a medium composed of: DME/HAM-F12 (1:1), 10% bovine serum, 2.5% fetal bovine serum, 40 mg/L gentamicin sulphate. The RCHT cell line was derived from the whole hypothalamus of an adult rat donor. Briefly, primary cultures of rat hypothalamus were treated with media conditioned by the UCHT1 rat thyroid cell line, a process that reportedly induces transformation *in vitro* (7, 8). RCHT cells grow in monolayers with culture medium conditioned with 10% bovine serum and 2.5% (proliferating medium) fetal calf serum, present a fibroblastoid morphology and under differentiation conditions, exhibit substantial process emission. Immunohistochemical studies on RCHT cells demonstrated the presence of neuronal markers MAP-2, synaptophysin, b-tubulin isotype III and NSE (neuron specific enolase). Glial markers were not evident. Dopaminergic markers such as tyrosine hydroxylase, DOPA decarboxylase and dopamine receptors were also evaluated, evidencing more intense expression when cultured under differentiation conditions. For experiments with SAL, the cells were incubated in the absence of bovine serum and phenol red for 120 min. Cell survival was measured by marking five 1 × 1 mm squares under the petri dish before seeding the cells. The cells were counted under contrast microscopy in the marked areas before and after the treatments. After the treatments, trypan blue was added to the plate before the second count. The incorporation of trypan blue into the cells and detachment of the cells from the plate were considered indicative of toxicity. The number of viable cells was then compared to the total counts prior to the incubation periods with the corresponding treatment.

RT-PCR. The expression of CuZn-superoxide dismutase, catalase, and glutathione peroxidase in the RCSN-3 cell line was studied by using the RT-PCR technique. The cells were incubated with 100 μM SAL; or 100 μM SAL and 100 μM DIC during 10 min before the extraction of total RNA. Total RNA was isolated by using the RNeasy Midi kit (QIAGEN). Five micrograms of the total RNA was used for the synthesis of a single strand DNA with the reverse transcriptase (RT) reaction. The RT reaction was performed using a Thermoscript RT-PCR system (Life Technologies) with oligo(dT)$_{20}$ as primers. The total RNA (1.5 μg) was incubated at 65°C for 5 min in DEPC-treated water before the addition of 4 μl 5× RT-buffer, 3 mM DDT, 40 U RnaseOUT, 1 μl DEPC water, 0.6 mM dNTP mix, and 15 U ThermoScript reverse transcriptase. The mixture was incubated at 25°C for 10 min, 50°C for 50 min and 85°C for 5 min before adding 2 U of RNase H, after which the mixture was incubated at 37°C for 20 min. The amplification of ssDNA of antioxidant enzymes was performed by the PCR reaction using the following primers 5′-CTCAGGAGAGCATTCCATCATTG-3′ (upstream) and 5′-ATCACACCACAAGCCAAGCG-3′ (downstream) designed from the cDNA sequence of rat CuZn-superoxide dismutase (11); 5′-CGCCTTTTTGCTTACCCAGAC-3′ (upstream) and 5′-AGAATGTCCGCACCTGAGTGAC-3′ (downstream) designed from rat catalase (12); 5′-ACAGTCCACCGTGTATGCCTTC-3′ (upstream) and 5′-TCTTGCCATTCTCCTGATGTCC-3′ designed from rat glutathione peroxidase (13); 5′-TTTGTGATGGGTGTGAACCACGAG-3′ (upstream) and 5′-CAACGGATACATTGGGGGTAGGAAC-3′ (downstream-1) and 5′-CCAGCATCAAAGGTGGAAGAATGG-3′ (downstream-2) designed from glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (14). The PCR reaction was performed in three steps: (i) 95°C for 5 min; (ii) 11 cycles at 95°C for 40 s, 65°C (decreasing the temperature one degree per cycle until 55°C) for 40 s and 72°C for 40 s; (iii) 28 cycles at 95°C for 45 s, 65°C for 40 s, 72°C for 50 s; (iv) one cycle at 72°C for 10 min. The PCR incubation contained 3 μl of RT incubation, 0.4 mM dNTP each, 3 mM $MgCl_2$, 2.5 μM primer, 5 μl 10× PCR-buffer (GibcoBRL), 29 μl H_2O and 2 U Taq polymerase (GibcoBRL). The portion of CuZn-SOD amplified by PCR spanned the region between bases 375-395 and 501-482, which resulted in a fragment of 129 bp (11); for catalase this was between bases 1143-1163 and 1422-1401, which resulted in a fragment of 280 bp (12); for glutathione peroxidase it was between bases 381-402 and 610-589, resulting in a fragment of 230 bp (13) and for GAPDH it was between bases 408-431 and 744-720, resulting in a fragment of 336 bp used as a housekeeping gene (14). PCR was performed at different numbers of cycles to estimate the differences in the level of expression of antioxidant enzyme mRNA between control and SAL- and SAL + DIC-treated RCSN-3 cells. The PCR products were electrophoresed on 2% agarose gels, stained with ethidium bromide and photographed. The photographs were scanned and analyzed in a computer by using a Scion Image software (Scion Corp., Gaithersburg, MD), in order to estimate the differences in the expression of glutathione peroxidase mRNA. The number of pixels estimated by the software was plotted against the number of cycles. GAPDH was used as control protein to demonstrate that the salsolinol effect on catalase and glutathione peroxidase mRNA expression was specific.

ESR studies. ESR measurements were carried out on a Bruker-ESP 300 spectrometer operating at the X-band (9.29 Ghz) and employing 100 kHz field modulation, with an ER 4116DM cavity in the perpendicular mode. Acquisition parameters were as follows: Microwave power 28 dB, scan time 40 s, time constant 20 ms, modulation amplitude 0.4 G and field range 50 G. The ESR spin-stabilization method (15), using Zn^{2+} to detect semiquinone radical formation in the peroxidative oxidation of catechol structures under air, was applied. All experiments were carried out at room temperature in acetate buffer (0.1 M, pH 6.0). The simulations were performed with the Simphonia Bruker program.

Theoretical calculations. Full geometry optimizations of SAL in spin-paired and free radical forms were carried out by AM1 methods. INDO calculations were done employing the open shell UHF option.

NMR spectroscopy. ^{1}H NMR spectra were obtained using a Bruker 500 spectrometer with standard probe and pulse sequence and an observation frequency of 499.65 MHz. Proton spectra were measured over a frequency range of 8000 Hz collecting 2000 transients with 65536 data points. The total time for data accumulation was 4 h. Spectra were processed with a broadening factor of 0.05 Hz zero filling once. Oxidation of SAL was performed in the NMR tube. Salsolinol was treated with a 5-fold excess of hydrogen peroxide in 0.1 M sodium dihydrogen phosphate buffer (pH 7.0) prepared with deuterium oxide, 20 μg LPO and incubated at room temperature for 1 h before starting the measurement. The final salsolinol concentration was 200 mM.

Tracer uptake into RCSN-3 and RCHT cells. The uptake of [^{3}H]dopamine into RCSN and RCHT cells was measured according to Arriagada *et al.* (20). [^{3}H]Dopamine (1 μCi) was added to each dish and the uptake was assessed after an incubation period of 1 min at 37°C in KREBS medium. The final concentration of dopamine was 100 μM. At the end of the uptake period, extracellular medium was removed and the cells were rapidly washed 5 times with 2 ml ice-cold KREBS medium to remove residual ^{3}H-tracers. Cell membranes were disrupted with 1 ml of 1% Triton X-100 and after 3 min incubation 900 μl of the cell/Triton X-100 extract was removed and analyzed for ^{3}H-tracer content by liquid scintillation counting. The results are expressed in nmole/mg protein/1 min.

Data analysis. All data were expressed as mean ± SD values. The statistical significance was calculated using unpaired Student's *t* test towards the control.

RESULTS

The Effect of SAL on RCSN and RCHT Cell Survival

We tested the toxicity of SAL toward RCSN-3 cultured dopaminergic neurons of rat substantia nigra. A concentration-dependent decrease in the survival of RCSN-3 cells was observed when treated with different concentrations of SAL (25 to 200 μM) (Fig. 1A). The concentration that decreased survival by 50% was found to be 208 μM salsolinol. In order to investigate whether this decrease was dependent on SAL oxidation to *o*-quinone and quinone metabolism we incubated the cells with 100 μM SAL in the presence of 100 μM dicoumarol. The decrease of survival rose significantly from 23% ($P < 0.001$) when the cells were incubated with 100 μM SAL, to 57% ($P < 0.001$) in the presence of 100 μM SAL and 100 μM dicoumarol (Fig. 1B). Incubation of RCSN-3 cells with 100 μM SAL and different concentrations of dicoumarol (25–100 μM) showed a dicoumarol-dependent survival decrease (Fig. 1C). Incubation of RCSN-3 cells with 100 μM dicoumarol alone decreased the survival by 9% (Fig. 1B). In order to determine whether dicoumarol-dependent decrease in survival of RCSN-3 cells treated with SAL was restricted to RCSN-3, we used non-substantia nigra cells as control. SAL or dicoumarol alone does not induce significant decrease of cell survival (3 and 5%, respectively). RCHT cells exhibited a slight decrease of survival of (9%) when cultured in the presence of SAL and dicoumarol (Fig. 1B). Interestingly, RCHT cells do not have an efficient and significant uptake of ^{3}H-dopamine as RCSN cells ($P < 0.001$) and in addition, the presence of 2 μM external nomifensine had no effect on tracer uptake (Table I).

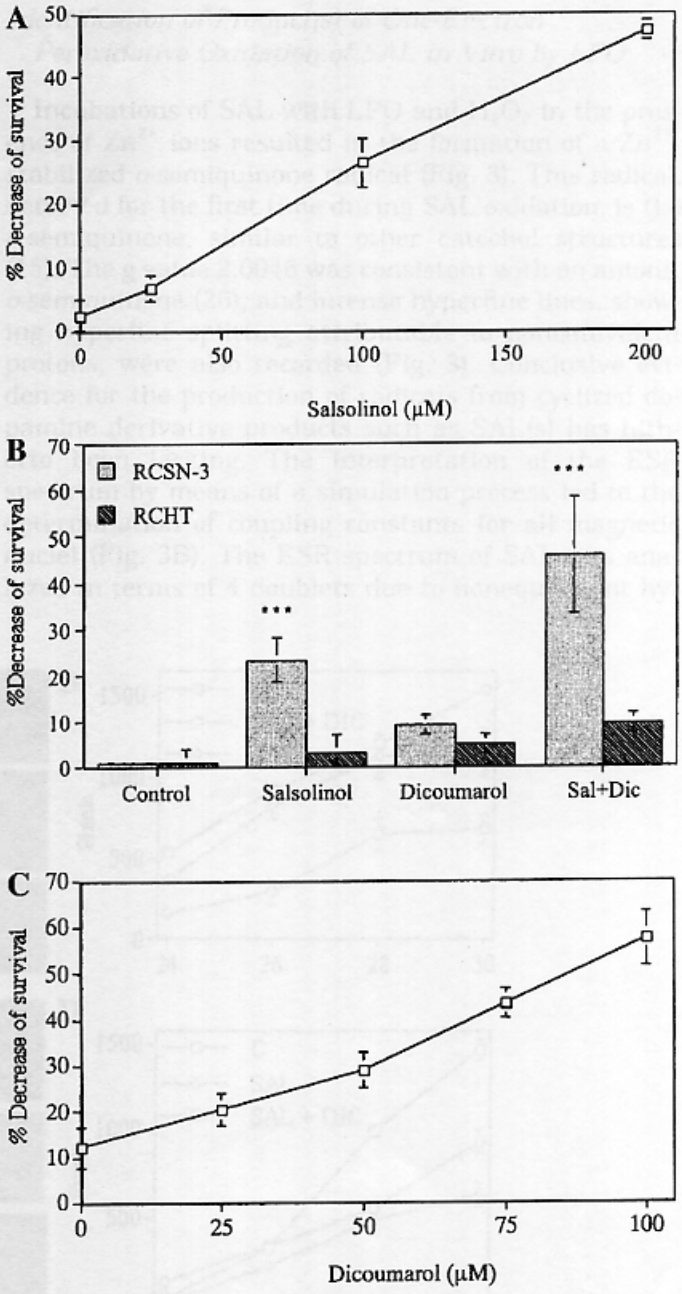


FIG. 1. The effect of SAL on RCSN-3 cell viability. (A) The cells were incubated with 0, 25, 100, and 200 μM salsolinol as described under Experimental Procedures. (B) The RCSN-3 and RCHT cells were incubated with 0 (control) or 100 μM salsolinol (SAL), 100 μM salsolinol and 50 μM dicoumarol, or 100 μM dicoumarol (DIC). The statistical significance of SAL ($P < 0.001$) and SAL-DIC ($P < 0.001$) was assessed by using Student's *t* test toward control. (C) The cells were incubated with 100 μM salsolinol in the presence of 0, 25, 50, 75, or 100 μM dicoumarol. The values are the means ± SD ($n = 3$).

Regulation of the Expression of Antioxidant Enzyme mRNA by SAL

The treatment of RCSN-3 cells with 100 μM SAL induced changes in the regulation of mRNA expression of the antioxidant enzymes catalase, CuZn-SOD and glutathione peroxidase (Fig. 2). The pattern of regulation seems to be different for these enzymes since the expression of CuZn-SOD mRNA was nearly unchanged (not shown), while catalase and glutathione peroxidase mRNA was significantly decreased (Fig. 2A, estimated as 76% ($P < 0.01$); and Fig. 2C, as 45% ($P < 0.001$); respectively). The treatment of RCSN-3 cells with 100

TABLE I

[^{3}H]Dopamine Uptake into RCSN-3 and RCHT Cells

	RCSN-3 cells	RCHT cells
	Uptake (pmole/mg/min)	
[^{3}H]Dopamine	2.3 ± 0.5	0.009 ± 0.004
[^{3}H]Dopamine + nomifensine	0.5 ± 0.3***	0.013 ± 0.013

Note. The uptake was measured as described under Materials and Methods. The statistic significance was measured by using Student's t test ($P^{***} < 0.001$).

μM SAL and 100 μM dicoumarol also decreased the mRNA expression of catalase and glutathione peroxidase, which was estimated as 45% ($P < 0.001$) and 65% ($P < 0.001$) of controls (Figs. 2A and 2B, respectively). The expression of GAPDH mRNA remained unchanged during the treatments detailed above (Fig. 1E).

Identification of Product(s) of One-Electron Peroxidative Oxidation of SAL in Vitro by LPO

Incubations of SAL with LPO and H_2O_2 in the presence of Zn^{2+} ions resulted in the formation of a Zn^{2+}-stabilized *o*-semiquinone radical (Fig. 3). This radical, detected for the first time during SAL oxidation, is the *o*-semiquinone, similar to other catechol structures (15). The g value 2.0046 was consistent with an anionic *o*-semiquinone (26), and intense hyperfine lines, showing hyperfine splitting attributable to nonequivalent protons, were also recorded (Fig. 3). Conclusive evidence for the production of radicals from cyclized dopamine derivative products such as SAL(s) has hitherto been lacking. The interpretation of the ESR spectrum by means of a simulation process led to the determination of coupling constants for all magnetic nuclei (Fig. 3B). The ESR spectrum of SAL was analyzed in terms of 4 doublets due to nonequivalent hy-

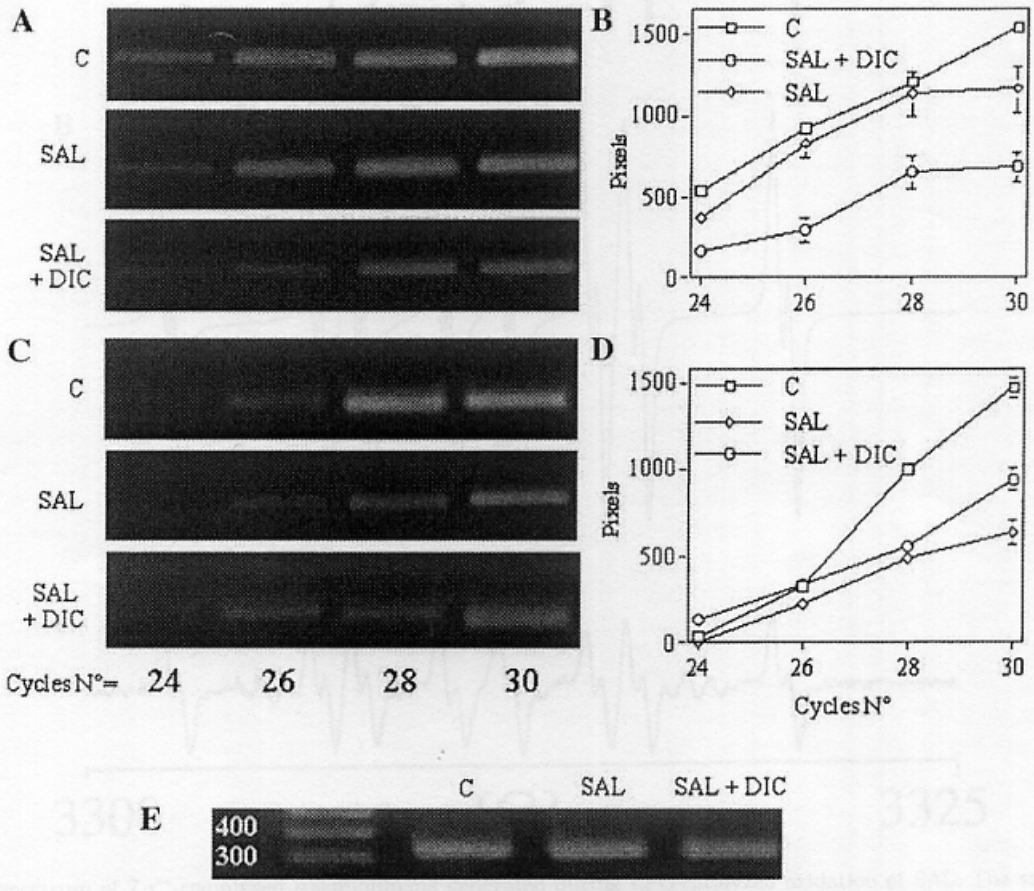


FIG. 2. Estimation of down-regulation of catalase (A, B) and glutathione peroxidase (C, D) mRNA in RCSN-3 cells treated with salsolinol in the presence or absence of dicoumarol by using RT-PCR. Total RNA extracted from RCSN-1 cells, incubated in the presence or absence of 100 μM salsolinol (SAL) or 100 μM salsolinol and 100 μM dicoumarol (SAL + DIC), was used for reverse transcriptase reactions. The PCR reactions at different cycle numbers were performed using primers specific for catalase (A) and glutathione peroxidase (C). (B) The estimation of differences in the expression of catalase was performed by scanning the gel shown in A. Values are means ± SD ($n = 3$). (D) The estimation of differences in the expression of glutathione peroxidase was performed by scanning the gel shown in C ($n = 3$). The amplification of GAPDH mRNA 336 bp (E) with RT-PCR using two different reverse primers was used as control. The statistical significance was determined by using Student's unpaired t test.

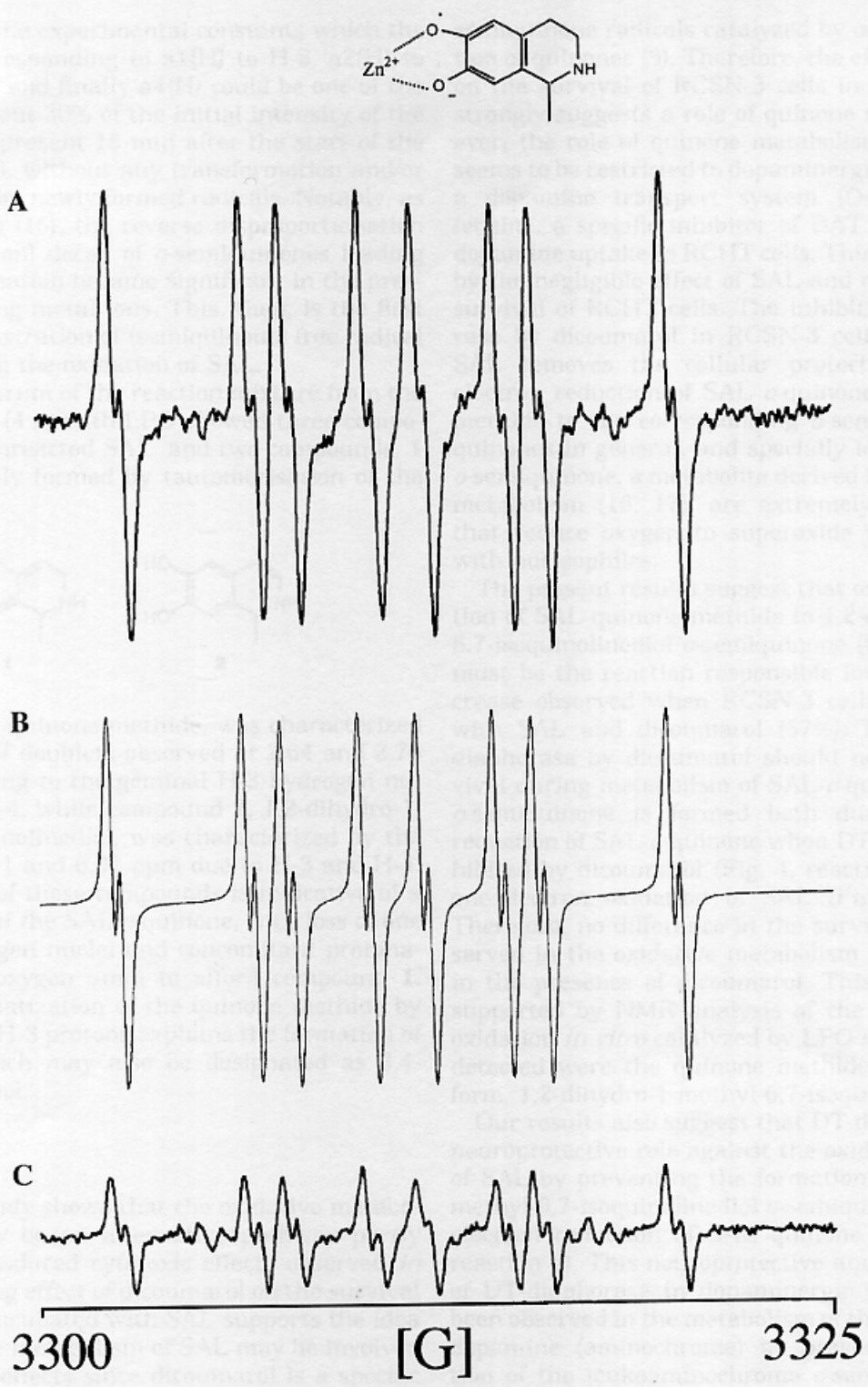
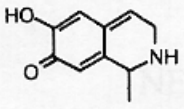


FIG. 3. ESR spectrum of Zn^{2+}-complexed *o*-semiquinone generated during LPO-catalyzed oxidation of SAL. The structure of SAL-*o*-semiquinone is presented at the top of the figure. (A) 1 min after starting the reaction by addition of 1 μM LPO to the incubation mixture containing 10 mM SAL, 0.86 mM H_2O_2 and 250 mM Zn^{2+} (0.1 M acetate buffer, pH 6.0). (B) Computer simulated spectrum. (C) 15 min after starting the reaction. Spectrometer conditions as described under Experimental Procedures.

drogen nuclei, whose constants are a1(H) = 7.82 G, a2(H) = 5.41 G, a3(H) = 4.25 G, and a4(H) = 0.32 G. In order to rationalize the experimental hyperfine constant, INDO calculation were done. We fully optimized the geometry for the electron-paired and anion radical molecules at AM1 level. The theoretical results are in

agreement with the experimental constants which the assignments corresponding to a1(H) to H-8, a2(H) to H-5 a3(H) to H-1 and finally a4(H) could be one of the H4 hydrogen. About 30% of the initial intensity of the signal were still present 15 min after the start of the reaction (Fig. 3C), without any transformation and/or superimposition by newly formed radicals. Notably, as previously shown (15), the reverse disproportionation (conproportionation) decay of *o*-semiquinones leading to *o*-quinone formation became significant in the presence of complexing metal ions. This, then, is the first conclusive demonstration of (semiquinone) free radical production during the oxidation of SAL.

The NMR spectrum of the reaction mixture from the oxidation of SAL (4 h) with LPO showed three components, namely: unreacted SAL, and two compounds, **1** and **2**, presumably formed by tautomerisation of the *o*-quinone.



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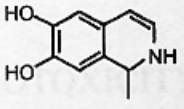

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Compound **1**, the quinone methide, was characterized by the doublets of doublets observed at 2.64 and 2.76 ppm, corresponding to the geminal H-3 hydrogen nuclei coupled to H-4, while compound **2**, 1,2-dihydro-1-methyl-6,7-isoquinolinediol, was characterized by the AB system at 6.91 and 6.97 ppm due to H-3 and H-4. The observation of these compounds is indicative of a tautomerisation of the SAL *o*-quinone, with loss of one of the H-4 hydrogen nuclei and concomitant protonation of the C-6 oxygen atom to afford compound **1**. Subsequent aromatization of the quinone methide by loss of one of the H-3 protons explains the formation of compound **2**, which may also be designated as 3,4-didehydrosalsolinol.

DISCUSSION

The present study shows that the oxidative metabolism of SAL may be an alternative pathway partly explaining SAL-induced cytotoxic effects observed *in vivo* (2). The strong effect of dicoumarol on the survival of RCSN-3 cells incubated with SAL supports the idea that the oxidative metabolism of SAL may be involved in its neurotoxic effects since dicoumarol is a specific inhibitor of DT-diaphorase (9) and incubation of the RCSN-3 cells with dicoumarol alone in the absence of SAL only had a weak effect on cell survival (9%) compared to SAL together with dicoumarol (57%). DT-diaphorase (EC 1.6.99.2, NAD(P)H: quinone (menadione) oxidoreductase) is unique among flavoenzymes since it reduces quinones to hydroquinones by transferring two electrons, preventing the formation of semiquinone radicals catalyzed by one-electron reduction of quinones (9). Therefore, the effect of dicoumarol on the survival of RCSN-3 cells incubated with SAL strongly suggests a role of quinone metabolism. However, the role of quinone metabolism in SAL toxicity seems to be restricted to dopaminergic neurons bearing a dopamine transport system (DAT), since nomifensine, a specific inhibitor of DAT, had no effect on dopamine uptake in RCHT cells. This idea is supported by the negligible effect of SAL and dicoumarol on cell survival of RCHT cells. The inhibition of DT-diaphorase by dicoumarol in RCSN-3 cells incubated with SAL removes the cellular protection against one-electron reduction of SAL *o*-quinone or SAL quinone methide to the corresponding *o*-semiquinones. Semiquinones in general, and specially leukoaminochrome *o*-semiquinone, a metabolite derived from DA oxidative metabolism (16, 17), are extremely reactive species that reduce oxygen to superoxide radicals, or react with nucleophiles.

The present results suggest that one-electron reduction of SAL quinone methide to 1,2-dihydro-1-methyl-6,7-isoquinolinediol *o*-semiquinone (Fig. 4, reaction 3) must be the reaction responsible for the survival decrease observed when RCSN-3 cells were incubated with SAL and dicoumarol (57%). Inhibition of DT-diaphorase by dicoumarol should not affect cell survival during metabolism of SAL-*o*-quinone since SAL-*o*-semiquinone is formed both during one-electron reduction of SAL-*o*-quinone when DT-diaphorase is inhibited by dicoumarol (Fig. 4, reaction 4) and during one-electron oxidation of SAL (Fig. 4, reaction 1). Therefore, no difference in the survival should be observed in the oxidative metabolism of SAL-*o*-quinone in the presence of dicoumarol. This idea is strongly supported by NMR analysis of the products of SAL oxidation *in vitro* catalyzed by LPO since the products detected were the quinone methide and its reduced form, 1,2-dihydro-1-methyl-6,7-isoquinolinediol.

Our results also suggest that DT-diaphorase plays a neuroprotective role against the oxidative metabolism of SAL by preventing the formation of 1,2-dihydro-1-methyl-6,7-isoquinolinediol *o*-semiquinone during one-electron reduction of SAL quinone methide (Fig. 4, reaction 6). This neuroprotective and antioxidant role of DT-diaphorase in dopaminergic systems has also been observed in the metabolism of the oxidized form of dopamine (aminochrome) by preventing the formation of the leukoaminochrome *o*-semiquinone radical (7, 17–20).

One-electron reduction of SAL quinone methide to *o*-semiquinone may explain the rise in RCSN-3 cell survival decrease from 23 to 57% when dicoumarol was added to the SAL-incubated cells. However, the 23% survival decrease observed when the cells were incubated solely with SAL can be explained by other mechanisms such as: (i) the formation of N-methyl(R)-SAL

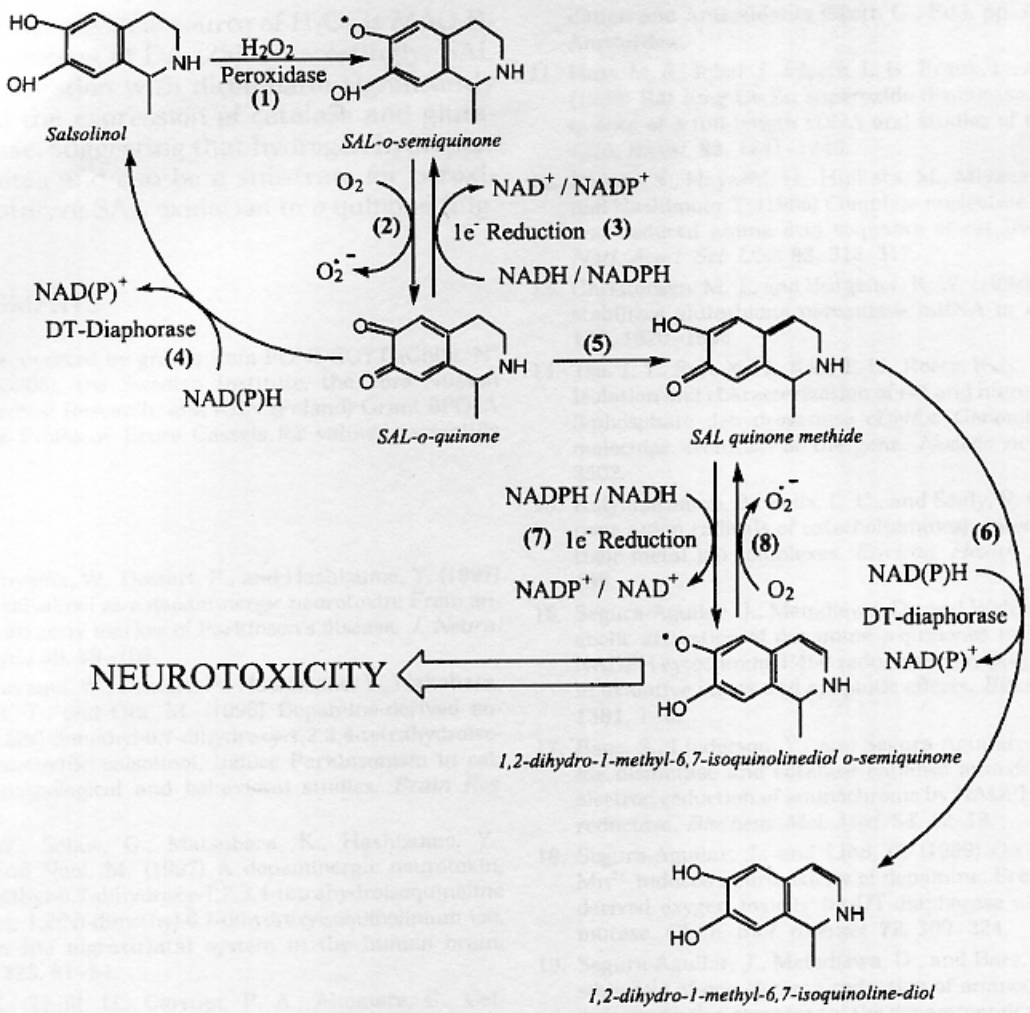

FIG. 4. Possible role of oxidative metabolism of salsolinol in its neurotoxic effects. Peroxidase catalyzes one-electron oxidation of SAL to (reaction 1), which autoxidizes to form SAL *o*-quinone (reaction 2). SAL *o*-quinone can be reduced by one-electron quinone reductases to SAL *o*-semiquinone radical (reaction 3) or by a two-electron reaction to SAL catalyzed by DT-diaphorase (reaction 4). However, SAL *o*-quinone tautomerizes to form SAL quinone methide (reaction 5), which can be two-electron reduced to 1,2-dihydro-1-methyl-6,7-isoquinolinediol (reaction 6) or one-electron reduced to *o*-semiquinone radical (reaction 7), which autoxidizes by reducing oxygen to superoxide radicals (reaction 8).

by N-methylation of SAL and its subsequent oxidation catalyzed by MAO-B to 1,2-dimethyl-6,7-dihydroxyisoquinolinium ion, which generates hydroxyl radicals (21); or (ii) the formation of SAL *o*-semiquinone by one-electron oxidation of SAL or one-electron reduction of SAL *o*-quinone (Fig. 4, reactions 1 and 3). Autoxidation of SAL *o*-semiquinone reduces oxygen to superoxide radicals that dismutate to form hydrogen peroxide, which is a precursor of hydroxyl radicals.

The formation of quinone methide and *o*-semiquinone radical determined by ^{1}H NMR and ESR, respectively, during *in vitro* oxidative metabolism of SAL catalyzed by LPO support the idea that oxidative metabolism may play a role in SAL-dependent survival decrease on RCSN-3 cells. LPO, a prototype for all mammalian peroxidases, catalyzes the one-electron oxidation of SAL to SAL *o*-quinone *via* formation of SAL *o*-semiquinone radical. It is of capital importance to stress that the presence of peroxidase activity was first demonstrated in substantia nigra, putamen and caudate nucleus (22). Furthermore, the highest peroxidase activity has been localized in the substantia nigra of the normal human brain (22). The peroxidative activity of prostaglandin H synthase, a membrane-associated enzyme that catalyzes oxidation of DA to *o*-quinone, cannot be ignored (23). In the absence of an identified brain-specific peroxidase enzyme, which can catalyze the oxidation of SAL, the possible involvement of cytochrome P450, a constituent hemoprotein ubiquitously distributed in brain, must also be taken into account. Cytochrome P4501A2 and other forms have been reported to oxidize DA to *o*-quinone (24). Thus, under oxidative stress conditions, with formation of H_2O_2, the peroxidative degradation of SAL could proceed very

efficiently. Another possible source of H_2O_2 is MAO-B-dependent metabolism of DA (25). Interestingly, SAL itself or in combination with dicoumarol significantly down-regulated the expression of catalase and glutathione peroxidase, suggesting that hydrogen hydroperoxide accumulates and can be a substrate for peroxidases, which catalyze SAL oxidation to *o*-quinone (Fig. 4, reaction 1).

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