Biochemical Pharmacology, Vol. 56, pp. 831–839, 1998.

ELSEVIER

ISSN 0006-2952/98/$19.00 + 0.00
PII S0006-2952(98)00228-7

Preferential Effects of Nicotine and 4-(*N*-Methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone on Mitochondrial Glutathione *S*-Transferase A4-4 Induction and Increased Oxidative Stress in the Rat Brain

Shripad V. Bhagwat, C. Vijayasarathy, Haider Raza, Jayati Mullick and Narayan G. Avadhani†*

LABORATORIES OF BIOCHEMISTRY, DEPARTMENT OF ANIMAL BIOLOGY, SCHOOL OF VETERINARY MEDICINE, UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA, PA 19104-6047, U.S.A.

ABSTRACT. We have investigated the *in vivo* effects of the tobacco-specific toxins nicotine and 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) on antioxidant defense systems in the mitochondrial, microsomal, and cytosolic compartments of rat brain, lung, and liver. Nicotine induced maximum oxidative stress in brain mitochondria, as seen from a 1.9-fold ($P < 0.001$) increase in thiobarbituric acid-reactive substance (TBARS) and a 2-fold ($P < 0.001$) increase in glutathione *S*-transferase (GST) A4-4 (also referred to as rGST 8-8) activities. These changes were accompanied by a 25–40% increase in reactive oxygen species and a 20–30% decrease in alcohol dehydrogenase activities. The 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-l-butanone-induced oxidative damage was apparent in the microsomal fraction of brain, lung, and liver, and it also increased 4-hydroxynonenal specific GST A4-4 activity in the brain and lung mitochondrial matrix fraction. The levels of microsomal thiobarbituric acid reactive substance, cytochrome P4502E1 activity, and reactive oxygen species were also increased significantly ($P < 0.001$) in all tissues. Both of these toxins induced the level of GST A4-4 mRNA in the brain, while they caused a marked reduction in the liver GST A4-4 mRNA pool. Additionally, the brain mitochondrial matrix showed a markedly higher level of 4-hydroxynonenal specific GST activity and mGST A4-4 antibody-reactive protein than did the cytosolic fraction. In conclusion, the present study provides evidence for the occurrence of GST A4-4 enzyme activity in mammalian mitochondria, in addition to demonstrating that both mitochondria and microsomes are intracellular targets for nicotine- and NNK-induced organ toxicity. BIOCHEM PHARMACOL **56**;7:831–839, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. nicotine; NNK; brain mitochondria; lipid peroxidation; glutathione *S*-transferase; mRNA induction; oxidative stress

Nicotine and its nitrosation product, NNK‡, are the major components of cigarette smoke that have been implicated in the development of cardiovascular diseases and lung cancers [1, 2]. Lipid peroxidation is associated with the pathogenesis of lung cancers, and an incidental observation is that the levels of lipid peroxides are invariably increased in smokers [3], thus implying a causal relationship. Previous studies have shown that nicotine induces oxidative stress in the rat gastric mucosa and pancreatic tissues *in vitro* as seen from increased lipid peroxidation [4, 5]. The effects of nicotine on enzymes of ethanol metabolism have also been studied [6]. Additionally, nicotine administration significantly inhibited hepatic and cerebral mitochondrial low-K_m ADH in the rat liver. Tissue-specific effects of nicotine were observed in another study, which showed that *in vivo* administration of nicotine induced the levels of P4502E1 in the brain but not in the liver [7]. It is known that P4502E1 generates ROS, thereby causing lipid peroxidation [8]. Oxidative stress to cells causes lipid peroxidation, an autocatalytic process that damages lipid-containing structures and generates reactive aldehydes such as MDA and 4-HNE, which covalently modify cellular macromolecules. In recent years, oxidative stress has been shown to be

* Visiting Scientist from the Department of Biochemistry, Faculty of Medicine and Health Sciences, United Arab Emirates University, P.O. Box 17666, Al Ain, United Arab Emirates.

† Corresponding author: Dr. Narayan G. Avadhani, 189E, School of Veterinary Medicine, Department of Animal Biology, University of Pennsylvania, 3800 Spruce St., Philadelphia, PA 19104-6047. Tel. (215)898-8819; FAX (215)898-9923; E-mail: narayan@vet.upenn.edu

‡ *Abbreviations:* ADH, alcohol dehydrogenase; CDNB, 1-chloro-2,4-dinitrobenzene; DCF, 2′,7′-diclorofluorescin; DCFdAc, 2′,7′-dichlorofluorescin diacetate; GST, glutathione *S*-transferase; 4-HNE, 4-hydroxynonenal; MDA, malondialdehyde; NNK, 4-(*N*-methyl-*N*-nitrosamino)-1-(3-pyridyl)-1-butanone; P4502E1, cytochrome P4502E1; ROS, reactive oxygen species; and TBARS, thiobarbituric acid reactive substance.

Received 5 February 1998; accepted 10 June 1998.

associated with age-related neurodegenerative disorders such as Alzheimer's disease [9, 10], Parkinson's disease [11], and amyotrophic lateral sclerosis [12]. 4-HNE, which cross-links proteins, including cytoskeletal proteins, has been shown recently to be cytotoxic to P19 neuroglial cells in culture [13].

Because lipid peroxidation is an important etiological factor in the pathogenesis of neurodegenerative and cardiovascular diseases, in the present study we tested the ability of nicotine and NNK to induce oxidative stress in target organs *in vivo*. Our results show that these two agents exert toxicological effects in both brain and lung, and, more importantly, induce GST A4-4 activity in brain mitochondria.

MATERIALS AND METHODS

Materials

(−)Nicotine hydrogen tartrate salt, 2-thiobarbituric acid, 5,5′-dithio-bis(2-nitro)benzoic acid (DTNB), CDNB, 4-HNE, NADPH, *p*-nitrophenol, *p*-nitrocatechol, and NAD+ were purchased from the Sigma Chemical Co. NNK was obtained from Chemsyn Science Laboratories. DCF was purchased from the Aldrich Chemical Co., and DCFdAc was obtained from Molecular Probes Inc. All other reagents and biochemicals used were purchased from the Fisher Scientific Co., or the Sigma Chemical Co.

Animals

Male Sprague–Dawley rats (150–200 g) were purchased from Harlan Sprague Dawley Inc. Rats were maintained at 20–25°, on a 12-hr light/12-hr dark cycle, with access to water and food *ad lib*. After 1 week of acclimatization, rats were administered intraperitoneally 1.6 mg/kg of (−)-nicotine hydrogen tartrate in saline or 1.6 mg/kg of NNK in warm water once daily for 10 consecutive days. Control rats received equal volumes of saline or water, respectively.

Subcellular Fractionation

Twenty-four hours after the last injection, rats were killed by CO_2 asphyxiation and were perfused with saline transcardially via the left ventricle. The liver, lung, and brain were quickly removed and washed with saline. All the tissues were homogenized in 10 vol. of H-medium (20 mM HEPES, pH 7.5, containing 70 mM sucrose, 220 mM mannitol, 2 mM EDTA, and 0.5 mg/mL of bovine serum albumin), and mitochondria were isolated by differential centrifugation essentially as described [14], except that brain mitochondria were purified further by Percoll density gradient centrifugation [15]. The mitochondrial preparations from all three tissues contained low levels (1–3%) of microsomal specific marker activities, glucose-6-phosphatase and NADPH cytochrome P450-reductase, and nearly 95–97% of mitochondrial specific cytochrome *c* oxidase and isocitrate dehydrogenase activities. The levels of various marker enzymes and the purity of mitochondrial isolates from different tissues were described recently [15, 16].

The mitochondrial and microsomal pellets were washed twice with the above buffer to reduce the levels of contaminating cytosolic proteins. Both mitochondrial and microsomal pellets were suspended in 50 mM potassium phosphate buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride and frozen quickly in dry ice. The cytosolic fractions were also frozen in aliquots in dry ice. All subcellular fractions were frozen at −70° until they were used for enzyme assays or measurements of oxidative stress parameters. For matrix preparation, mitochondria were sonicated and centrifuged at 120,000 *g* for 1 hr. The resulting supernatant matrix fraction and the cytosolic 100,000 *g* supernatant were used as the source of the mitochondrial and cytosolic GST enzymes, respectively.

Enzyme Assays

GSH levels in the cytosolic and mitochondrial fractions were determined as protein-free sulfhydryl content using Ellman's reagent [17] or by the method of Tietze [18] using deproteinized extracts. Lipid peroxidation was estimated by measuring TBARS levels [19]. Although this method has been commonly used to measure the level of lipid peroxide-derived MDA, TBA is known to react with other lipid and non-lipid components at significantly lower affinity. The cytosolic and mitochondrial GST activities were measured using CDNB or 4-HNE as substrates, according to the method of Habig *et al.* [20] or Alin *et al.* [21], respectively. The microsomal GST activities were assayed as described earlier [22]. Both cytosolic and mitochondrial ADH activities were assayed by a spectrophotometric method, using ethanol as the substrate [23]. P4502E1 activities in microsomes and mitochondria were determined using *p*-nitrophenol as a substrate [24]. The amount of ROS was measured in tissue homogenates by a fluorescence spectrophotometric method, using DCFdAc as a probe [25, 26]. The protein concentration was estimated by the dye-binding method of Bradford [27], using bovine serum albumin as a standard. The statistical analysis of the data was carried out by Student's *t*-test, using average and standard deviation values derived from six to eight enzyme activity measurements.

Western Blot Analysis

Both cytosolic and mitochondrial proteins were subjected to SDS–PAGE on 14% gels as described [28], and the separated proteins were electroblotted onto nitrocellulose membranes [29]. The membranes were immunostained with antisera to either total α class of GST from rat liver [30] or antibody raised against recombinant mGST A4-4 [31]. Both of these antibodies were provided by Dr. Yogesh Awasthi.

Northern Blot Analysis

Total RNA was extracted from rat liver, lung, and brain tissues by the acid guanidinium thiocyanate method [32]. Thirty micrograms of total RNA was separated on a 1.2% agarose formaldehyde gel, blotted onto Nylon membrane (Nytran, S&S), and hybridized to a GST A4-4 cDNA probe or a DNA sequence of 75 nucleotides in length, corresponding to the active site region of GST A4-4 as described [33]. The probes were labeled with ^{32}P by random-primed oligolabeling. The amount of RNA loaded was normalized by reprobing the Northern blot with a ^{32}P-labeled DNA probe specific for 18S rRNA.

RESULTS

Figure 1 shows the effects of nicotine and NNK on the mitochondrial and cytosolic GSH pools and also the MDA contents of the mitochondrial and microsomal membrane fractions from different tissues. It is seen that on a per milligram of protein basis, the liver cytosol contained about a 2- to 5-fold higher level of GSH than the mitochondrial membrane-matrix compartment. Results (Fig. 1A) show that nicotine administration reduced the liver cytosolic GSH by about 50% ($P < 0.001$), while the lung and brain cytosolic GSH levels were not affected. Additionally, both nicotine and NNK treatments reduced the mitochondrial GSH in the lung by 40% ($P < 0.001$) and 30% ($P < 0.001$), respectively, whereas they had no effect on brain and liver mitochondrial GSH. Similarly, nicotine administration caused a 1.3- to 1.4-fold ($P < 0.001$) increase of TBARS, which predominantly consists of lipid peroxide-derived MDA, in the hepatic mitochondria and microsomes as well as the lung microsomal compartments. NNK, on the other hand, had no significant effect on the level of TBARS in these fractions, except in the brain microsomes where the level was increased significantly. A significant effect of nicotine was found in the brain, where it caused about a 50% ($P < 0.001$) increase in the TBARS and a nearly two-fold ($P < 0.001$) increase in the mitochondrial TBARS level. These results demonstrated that the two structurally related tobacco toxins affect the three target tissues to different extents.

GST is an important indicator of tissue resistance to toxicity induced by different xenobiotics. Therefore, we measured the GST levels in the mitochondrial, microsomal, and cytosolic compartments of the three target tissues from rats treated with nicotine or NNK. Results in Fig. 2A show that, using CDNB as a substrate, the liver and brain cytosolic fractions of untreated rats had 6- and 4-fold higher activity, respectively, than that detected in the lung cytosolic fraction. Results also demonstrated that neither nicotine nor NNK had any effect on the cytosolic CDNB conjugation activity in all three tissues. However, the liver and brain mitochondrial activities for CDNB conjugation were selectively and significantly inhibited 25–30% ($P < 0.001$) by NNK and nicotine, while the lung mitochondrial GST activity was reduced marginally (17%) by both agents. The GST-dependent 4-HNE metabolism in the cytosol of all three tissues was not affected significantly by either nicotine or NNK. However, the mitochondrial

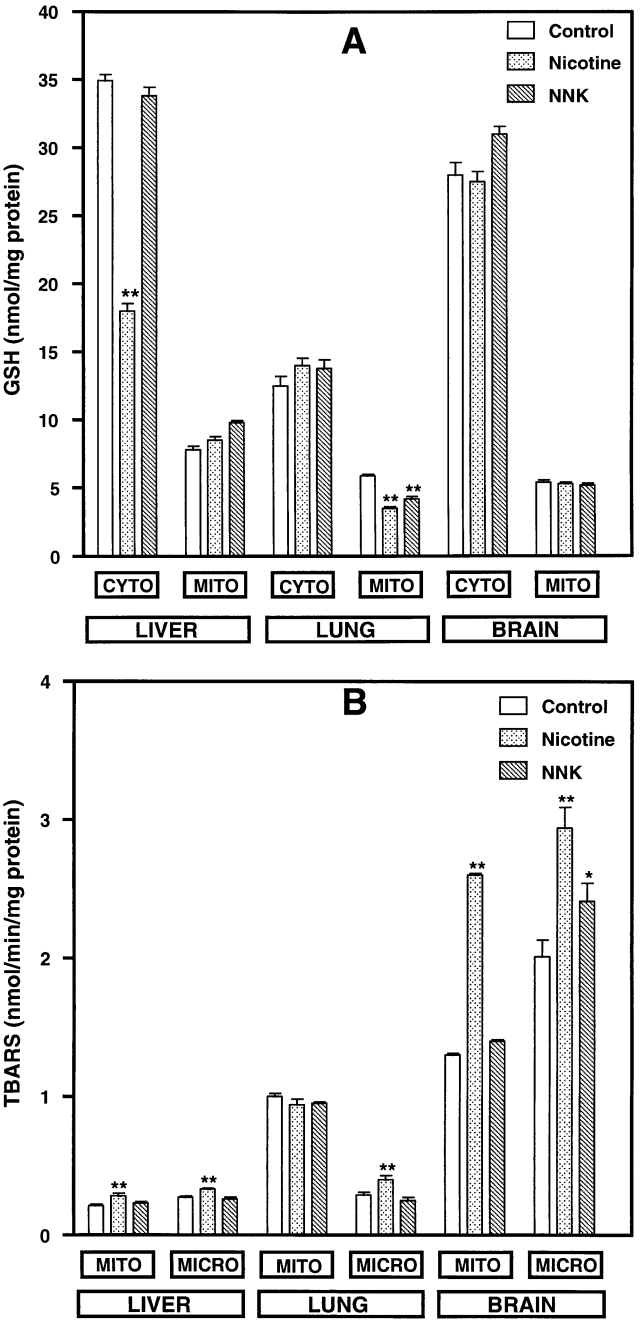

FIG. 1. Effects of nicotine and NNK on rat hepatic, pulmonary, and cerebral GSH and TBARS. Cytosol, mitochondria, and microsomes from treated and untreated rat liver, brain, and lung were isolated as described under Materials and Methods. Cytosol and mitochondria were assayed for total GSH pool (A), and mitochondria and microsomes were assayed for TBARS (B). Assays were carried out in quadruplicate with samples derived from two batches of animals (three rats in each batch). Values are means ± SD. Bars marked with two asterisks indicate a significant difference from the controls with a P value of < 0.001, and a single asterisk indicates a P value of < 0.005.

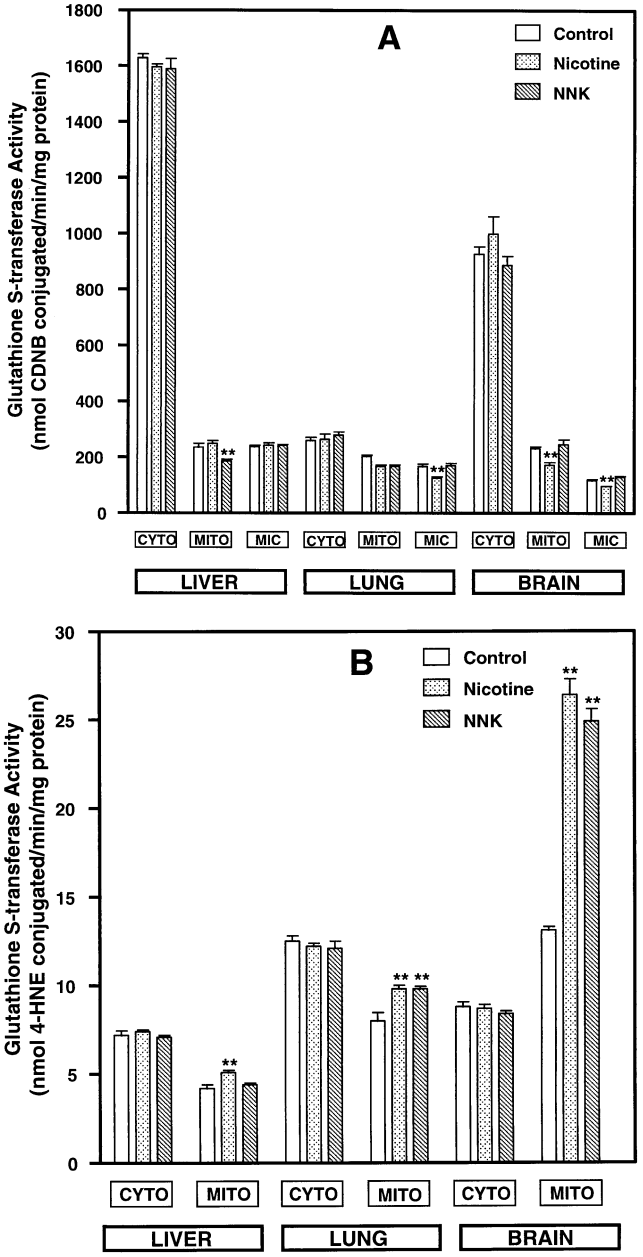

FIG. 2. Effects of nicotine and NNK on GST activities in subcellular fractions of different tissues. Subcellular fractions (mitochondria, microsomes, and cytosol) from liver, lung, and brain of treated and untreated rats were isolated as described in Materials and Methods, and GST activities were assayed using CDNB (A) or 4-HNE (B) as a substrate. Assays were carried out in quadruplicate with samples from two batches of animals (three rats in each batch). Values are means ± SD. Bars marked with two asterisks reflect a significant difference from the controls with *P* values of < 0.001.

4-HNE metabolic patterns in these tissues were quite different in that both of these agents induced none to marginal activity in liver and lung mitochondria, while both induced brain mitochondrial 4-HNE activity nearly two-fold ($P < 0.001$).

4-HNE is known to affect brain mitochondrial respiratory-coupled oxidative phosphorylation [34, 35]. However,

TABLE 1. GSTA4-4 activity in intact and disrupted rat brain mitochondria

Source of enzyme	GST A4-4 activity (nmol 4-HNE conjugated/mg protein/min)
Intact mitochondria	0.0
Sonicated mitochondria	3.8 ± 0.22
Freeze and thawed mitochondria	8.7 ± 0.46
Mitochondrial matrix	12.8 ± 0.64
Mitochondrial inner membrane	0.22 ± 0.01

Brain mitochondria were isolated by Percoll density gradient centrifugation. Submitochondrial fractions were prepared as described under Materials and Methods. The mitochondrial suspension was sonicated for 10 sec using a Branso Sonifer Cell Disruptor 2000 or subjected to four repeated cycles of quick freezing in dry ice acetone and thawing at 37°. Assays were carried out in triplicate using samples from two different preparations (N = 6). Values are means ± SEM.

currently, there is no information on the mitochondrial 4-HNE-specific GST in any of the mammalian tissues. Therefore, the mitochondrial membrane matrix location of the GST A4-4 enzyme was ascertained further by assaying mitochondria with intact and disrupted membranes, as well as different submitochondrial fractions. Results in Table 1 demonstrate that intact rat brain mitochondria yielded negligible GST A4-4 activity, while mitochondria subjected to sonic disruption or repeated freezing/thawing yielded activities in the range of 4–9 nmol/mg of protein. Furthermore, the brain mitochondrial matrix fraction showed the highest activity in the range of about 13 nmol/mg of protein. These results demonstrate that the mitochondrial-associated 4-HNE activity is indeed localized in the mitochondrial matrix fraction, and requires physical disruption of the mitochondrial membrane envelope to be released. Although not shown, the GST A4-4 activities associated with the liver and lung mitochondria were fully apparent only after disruption of the mitochondrial membrane, suggesting impermeability of the substrate, 4-HNE, through the mitochondrial inner membrane.

To gain further insight into the nature of mitochondrial GST-dependent 4-HNE metabolism, and its induction by NNK and nicotine, we carried out Northern blot analysis of RNA and Western blot analysis of proteins from the three tissues, using rat GST A4-4 specific cDNA and mGST A4-4 antibody probes, respectively. Results of Northern blot analysis (Fig. 3A) show that the cDNA probe hybridized to a single 1-kb mRNA from the liver as against two RNAs of 1 and 0.8 kb from the lung and brain total RNAs. The relative abundance of GST A4-4 mRNA appears to be several-fold higher in the lung than in the liver and brain. It is noteworthy that both nicotine and NNK treatments reduced the steady-state levels of the 1-kb mRNA in the liver, while they induced the levels of both the 1.0 and 0.8 kb mRNA species in the brain. These treatments also marginally increased the relative abundance of both mRNAs in the lung. The results, therefore, suggest a surprising tissue-specific induction or suppression of GST A4-4 mRNA expression by nicotine and NNK. Since the rat

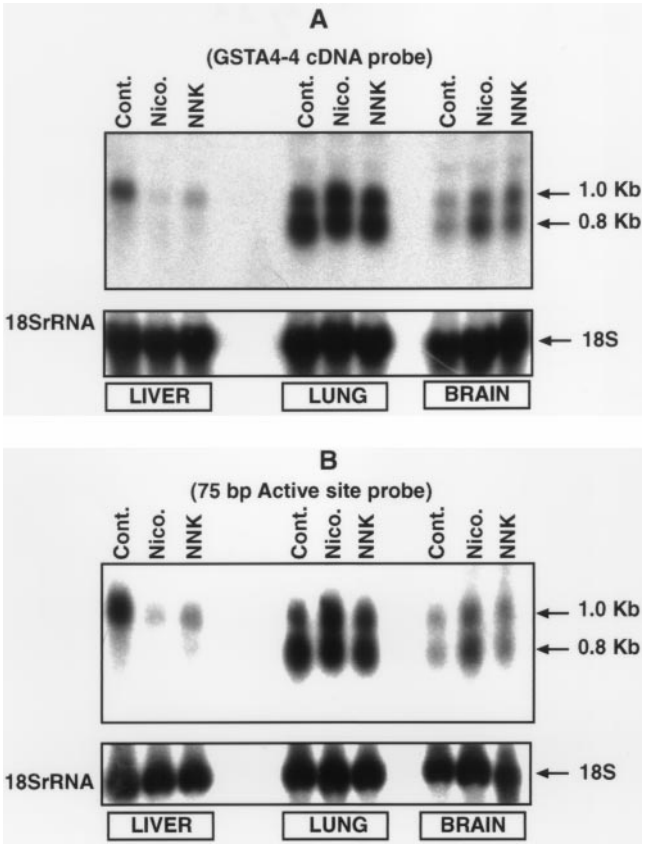

FIG. 3. Northern blot analysis of RNA from different tissues. Total RNA (30 μg each) from different treated and untreated rat tissues was resolved on denaturing agarose gel and subjected to Northern blot hybridization, using (A) nearly intact rat GST A4-4 cDNA probe, and (B) the 75 bp sequence from the substrate binding region of the protein, which is known to be highly specific for GST A4-4. Blots were hybridized with 1–2 × 10^6 cpm/mL of the ^{32}P-labeled probes under moderate stringency conditions, as described in Materials and Methods. The stripped blots were rehybridized with the 18S ribosomal probe. The blots in A and B are from parallel gels run under the same conditions.

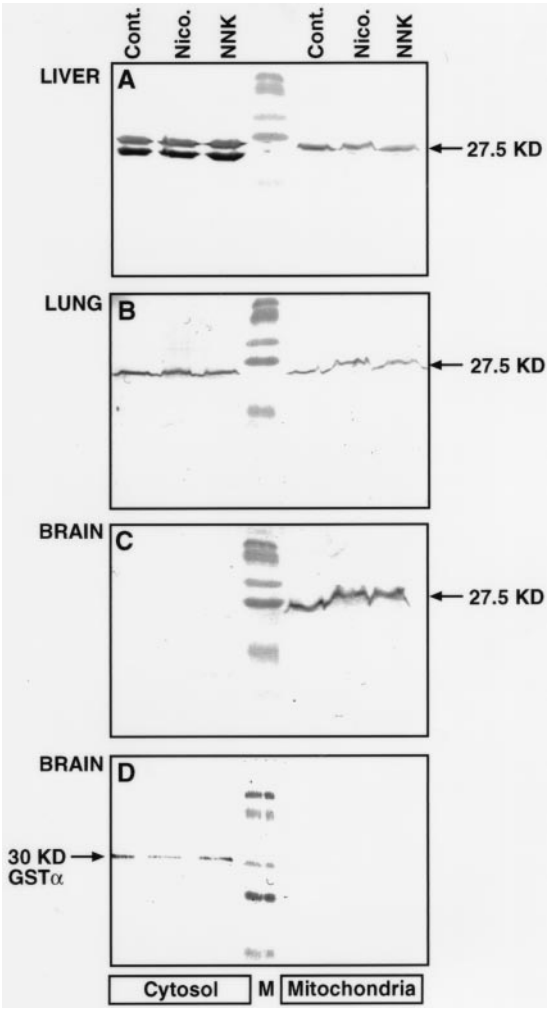

FIG. 4. Western immunoblot analysis of cytosolic and mitochondrial proteins from various rat tissues. Mitochondrial and cytosolic proteins (100 μg each) from the liver, lung, and brain from both treated and untreated rats were subjected to Western blot analysis as described in Materials and Methods. Blots in A–C were probed with antibody to mGST A4-4, while the blots in D (proteins from treated and untreated brain) were probed with GST α antibody. The positions of rat GST A4-4 (~25 kDa) and GST α (30 kDa) are indicated. The blots were quantitated using a Bio-Rad Image Analysis System. In lanes marked M, prestained molecular weight markers were loaded.

GST A4-4 mRNA is reported to show extensive homology to the mouse α family GST genes, the specificity of hybridization shown in Fig. 3A was further ascertained using a 75-bp DNA sequence from the active site encoding region of the rat GST A4-4 cDNA [31]. This sequence provides a highly specific and discriminating probe, because the GST A4-4 family members are believed to contain a unique active site sequence [31]. Results in Fig. 3B show that, similar to the intact cDNA probe, the 75-bp active site probe hybridized with both the 1- and 0.8-kb mRNAs characteristic of GST A4-4. Additionally, the mRNA pools demonstrated the characteristic NNK- and nicotine-mediated reduction in the liver, and induction in the brain, as seen with the more intact cDNA probe (Fig. 3A). These results confirm the identity of the GST mRNAs, and also suggest the occurrence of two different GST A4-4 mRNA species in the lung and brain.

The results of Western blot analysis in Fig. 4 using rat GST α and mGST A4-4 antibodies show that the liver cytosol from both the treated and untreated rat livers contained a 27.5-kDa immunoreactive protein of relatively lower abundance, in addition to a 25-kDa species of higher abundance. Based on the apparent size, the latter species may be the previously reported mGST A4-4 [31]. Thus, the 27.5-kDa species observed in this study appears to be quite different from the previously described 4-HNE-specific GSTs from the mouse or rat. The mitochondrial fractions, on the other hand, showed only the 27.5-kDa immunoreactive species. Neither nicotine nor NNK had any significant effect on the intensities of the GST A4-4 antibody reactive bands in either of the cell compartments. As seen from Fig. 4B, the cytosolic and mitochondrial fractions of both treated and untreated lung contained only the 27.5-

kDa immunoreactive protein species. Surprisingly, the cytosolic fractions of both treated and untreated rat brain failed to show a clearly detectable antibody-reactive protein, while the mitochondrial fractions from these tissues showed the 27.5-kDa antibody-reactive species. Quantitation of immunoreactive bands using the Bio-Rad Molecular Imager System (GS-525) indicated 50–80% higher abundance of proteins in the NNK- and nicotine-treated samples. In support of the GST activity data in Fig. 2B, these results provide evidence for the occurrence of a 27.5-kDa GST A4-4 antibody-reactive protein in mitochondria of all three tissues studied.

The presence of mGST A4-4 antibody-reactive protein in the mitochondria of the rat brain, but not in the cytosol, in Fig. 4C was indeed surprising. Thus, to further document these results, and also to determine if the mitochondrial antibody-reactive proteins observed are different from the previously characterized α form of GSTs [36], a companion blot as in Fig. 4C (containing brain cytosolic and mitochondrial proteins) was probed with antibody to total rat cytosolic GST α. As seen from Fig. 4D, the pattern with GST α-antibody was distinctly different in that only the cytosolic protein, and not the mitochondrial protein showed cross-reactivity. Furthermore, the GST α antibody-reactive protein from the brain cytosol migrated behind the 29-kDa marker protein, whereas the mGST A4-4 antibody-reactive proteins migrated differently on the SDS gels, with an apparent molecular mass of 27.5 kDa. These results provide further evidence for the mitochondrial location of a majority of the GST A4-4 antibody-reactive protein in the brain.

The levels of oxidative stress induced by nicotine and NNK in different tissues were assessed by measuring the ADH and P4502E1 activities in different cell compartments and the levels of ROS in whole tissue homogenates of treated and untreated rats. The results in Fig. 5A show that both the cytosolic and mitochondrial ADH activities were inhibited significantly in the liver, lung, and brain of nicotine-administered rats. The extent of inhibition varied between 30 and 60% ($P < 0.001$) and was found to be maximum in the lung cytosol and mitochondria. On the other hand, NNK administration had a generally smaller effect on both cytosolic and mitochondrial ADH activities of liver and lung. The brain mitochondrial ADH activity was inhibited significantly by 30% ($P < 0.001$). Furthermore, *P*-nitrophenol hydroxylase (pNPH) activity, a known marker for P4502E1, was detectable only in the liver mitochondria, while the microsomal fractions from all three tissues contained varied levels of activities (Fig. 5B). Nicotine administration marginally induced pNPH activity ($P < 0.001$) in rat liver mitochondria, whereas NNK had no appreciable effect. Interestingly, both nicotine and NNK treatments induced pNPH activities in the rat liver and lung microsomes by 2 to 3.5-fold ($P < 0.001$). Results in Fig. 5C also show that the total tissue ROS level was increased by about 25–40% ($P < 0.001$) in brain, lung, and liver of nicotine-administered animals. Additionally, NNK treatment also increased the ROS level by about 40% ($P < 0.001$) only in the liver; it had no effect on the brain and lung ROS. These results collectively suggest that both nicotine and NNK significantly induce oxidative stress in different compartments of all three tissues, although the most notable effects are on the brain mitochondria.

DISCUSSION

The present study demonstrates that nicotine and NNK induce oxidative stress *in vivo* in rat brain, lung, and liver, as indicated by increased TBARS, ROS, and P4502E1 activity. In two previous studies, Wetscher *et al.* [4, 5] demonstrated that nicotine induces oxidative stress *in vitro* in rat pancreatic tissue and esophageal mucosa. To our knowledge, there have been no reports on the ability of nicotine, an addictive substance, to induce oxidative stress in target organs *in vivo*. Some addictive substances, such as cocaine and ethanol, induce oxidative stress *in vivo* by depletion of GSH or by increased lipid peroxidation, both of which affect mitochondrial function [37–39]. For this reason, we studied a number of different oxidative stress parameters in the lung, brain, and liver of rats administered nicotine and NNK. Surprisingly, both of these agents increased the levels of TBARS in both the mitochondrial and microsomal compartments, whereas the 4-HNE-specific GST activity was induced only in the mitochondrial and not in the cytosolic fractions. It is generally believed that oxidative stress is associated with depletion in GSH level, increased lipid peroxidation, and increased GST activities [37, 38, 40]. The results of the present study, however, suggest that both nicotine and NNK affect the various GST isoenzymes differently, albeit to different extents. Results in Fig. 2 show that both nicotine and NNK marginally inhibited GST activities measured with CDNB as a substrate, while they induced GST activities determined using 4-HNE as the substrate.

4-HNE is a membrane lipid peroxidation product, which is a physiological substrate for GST A4-4 and also its specific inducer [21, 41]. It is therefore likely that the induction of GST A4-4 activity by nicotine is due to increased production of 4-HNE. Thus, if one adds up the increase in lipid peroxidation measured as TBARS and GST-dependent 4-HNE activity, the total amount of lipid peroxides formed in each of the target tissues will be much higher, particularly in the brain, where the latter activity is induced nearly two-fold. Efforts are underway to quantitate the individual lipid peroxide products in different nicotine- and NNK-treated tissues.

The GST-dependent 4-HNE metabolism (Fig. 2B) indicates that the mitochondrion is the major intracellular target for both nicotine- and NNK-induced toxicity, with the brain being the most affected target followed by the lung and liver. Recently, Keller *et al.* [35] demonstrated that 4-HNE impairs glutamate transport and mitochondrial functions in synaptosomes. Previous studies [13, 42] demonstrated that 4-HNE is cytotoxic and cross-links cytoskel-

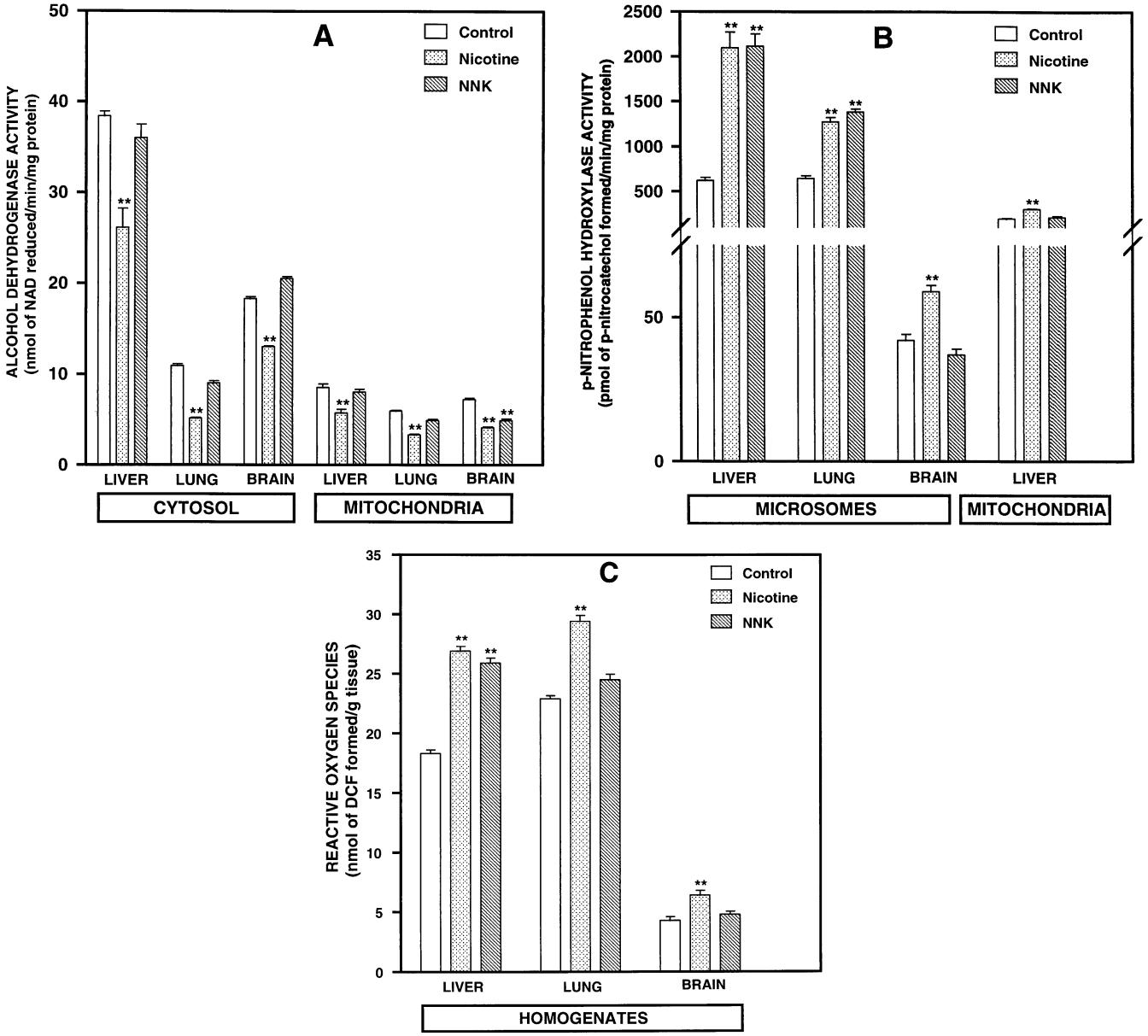

FIG. 5. Effects of nicotine on oxidative defense systems and ROS. Subcellular fractions were isolated from treated and untreated rat tissues as described in Materials and Methods. Alcohol dehydrogenase activity is expressed as nmol NAD reduced/min/mg of protein (A). *p*-Nitrophenol hydroxylase activity is expressed as pmol *p*-nitrocatechol formed/min/mg protein (B). ROS were assayed fluorometrically as nmol DCF formed/g of tissue (C). All assays were carried out in quadruplicate with samples from at least two batches of animals (three rats in each batch). Values are means ± SD. Bars with two asterisks indicate a significant difference from the controls with $P < 0.001$.

etal proteins in P19 neuroglial cultures. Moreover, the age-related neurodegenerative diseases are associated with increased lipid peroxidation and ROS [10–12]. In view of our findings on the preferentially higher level of nicotine-mediated lipid peroxidation in the brain, it appears highly likely that this tobacco alkaloid directly contributes to neurodegeneration.

Recently, an enzyme specific for the metabolism of 4-HNE (an ortholog of mGST A4-4) was purified from bovine lung microvessel endothelial cells [43]. Although 4-HNE is known to affect various mitochondrial functions as well, there is currently no information on its mitochondrial location. The present results, therefore, provide the first insight on the mitochondrial location of this important detoxifying enzyme. Results of control experiments in Table 1 show that the intact, well-washed mitochondria exhibited negligible 4-HNE conjugation, while disruption of mitochondrial membrane by freezing/thawing or sonication resulted in high activity, suggesting that the enzyme is indeed localized within the mitochondrial membrane compartment. A highly interesting observation is that both in terms of enzyme activity profile (Fig. 2B) and western immunoblot analysis (Fig. 4) brain mitochondria contain higher levels of GST A4-4 homologous enzyme than does

brain cytosol. Even in the liver and lung where the cytosolic contents are significantly higher (Figs. 2 and 4), the mitochondrial specific contents amounted to about 60% of the cytosolic specific contents. In view of the mitochondrial purity criteria described in Materials and methods, these results dispel any possibility that cytosolic contamination is the basis for the observed mitochondrial activities.

Another important observation of this study relates to the specific effects of nicotine and NNK on the brain GST A4-4 mRNA pool (Fig. 3). In support of results in Figs. 2 and 4, there was a 10- to 20-fold higher level of GST A4-4 mRNA in the treated brain, as compared with no significant effects in the lung and several-fold reduced levels in the liver. These results therefore suggest that similar to polycyclic aromatic hydrocarbons and polychlorinated biphenyls, nicotine and NNK may have additional functions in modulating the transcriptional activities of some of the target genes. However, the diametrically opposing effects of NNK and nicotine on the GST A4-4 mRNA level in the liver are intriguing. It is noteworthy that the reduced steady-state levels of GST A4-4 mRNA are not consistent with the levels of antibody-reactive proteins (Fig. 4) in the mitochondrial and cytosolic compartments or the GST A4-4 activity levels (Fig. 2B) in the liver. It is possible that the steady-state mRNA levels even under the reduced conditions are sufficient for maintaining normal protein levels, possibly through increased translational activity. Alternatively, other GST isoforms with overlapping substrate specificities are induced by these agents, which, in turn, help maintain the near normal levels of 4-HNE hydroxylase activity.

Previous studies on Northern blot hybridization with mouse liver RNA show a single 1.2-kb mRNA [31]. In support of these observations, our results also showed a single 1-kb mRNA in the liver, although two mRNAs of 1.0 and 0.8 kb were observed in the lung and brain. Recent studies by Zimniak *et al.* [31] on the hybridization of genomic DNA with the GST A4-4 active site specific 75 bp DNA probe suggested the presence of two distinct genes for this isoform. In view of these results, it is likely that the two mRNA species characterized in the present study may represent two different gene products, although the possibility of a differently processed single gene product cannot be ruled out. Similarly, the precise nature of the mitochondrial 27.5-kDa protein detected in this study remains unclear. It may either represent a distinct gene product or be encoded by the same gene that encodes the cytosolic GST A4-4 by using an alternate upstream translation site. Studies are in progress to test these possibilities.

It is known that ethanol is metabolized by both cytosolic and mitochondrial alcohol dehydrogenase isoenzymes in liver. Additionally, it has been shown that cannabis or tobacco extract inhibits the liver and brain mitochondrial ADH activities [6]. In extension of these studies, our results show that both nicotine and NNK inhibit the mitochondrial as well as the cytosolic ADH activities in all of the three target tissues studied. It is therefore likely that nicotine may help potentiate ethanol toxicity, particularly in the brain where the ADH inhibition is more pronounced.

In summary, the present study demonstrated that nicotine and NNK induce oxidative stress *in vivo* in brain and lung, which are the important target organs for nicotine and NNK, respectively. Additionally, our results suggest that increased mitochondrial GST A4-4 activity in both of these tissues may represent a compensatory pathway for countering nicotine- and NNK-mediated lipid peroxidation and oxidative injury.

This investigation was supported, in part, by National Institutes of Health Grants CA-22762 and GM34883-13. We thank Dr. Yogesh C. Awasthi for the gift of GST antibodies. We also thank Drs. Hasan Mukhtar and Yogesh Awasthi for their insightful and critical review of the manuscript.

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