JPET Fast Forward. Published on February 17, 2005 as DOI: 10.1124/jpet.104.080242

Cyclooxygenase-2 is an Obligatory Factor in Methamphetamine-Induced Neurotoxicity

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Running title: COX-2 and methamphetamine neurotoxicity

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Number of text pages: 25
Number of tables: 0
Number of figures: 6
Number of references: 42
Words in abstract: 199
Words in introduction: 355
Words in discussion: 1507

ABBREVIATIONS: 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP; 5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole, SC-560; N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide, NS-398; area under the curve, AUC; CCAAT/enhancer-binding protein, C/EBP; cyclooxygenase, COX (-1,-2, and -3 isoforms); dopamine, DA; methamphetamine, METH; prostaglandin E_2, PGE_2.

Journal section assignment: Neuropharmacology or toxicology

ABSTRACT

Methamphetamine causes persistent damage to dopamine nerve endings of the striatum. The mechanisms underlying its neurotoxicity are not fully understood but considerable evidence points to oxidative stress as a probable mechanism. A recent microarray analysis of gene expression changes caused by methamphetamine revealed that cyclooxygenase-2 (COX-2) was induced along with its transcription factor CCAAT/enhancer-binding protein (Thomas et al., 2004). We report presently that methamphetamine increases striatal expression of COX-2 protein. Cyclooxygenase-1 (COX-1) expression was not changed. Mice bearing a null-mutation of the gene for COX-2 were resistant to methamphetamine-induced neurotoxicity. COX-1 knockouts, like wild-type mice, showed extensive dopamine nerve terminal damage. Selective inhibitors of COX-1 (5-(4-chlorophenyl)-1-(4-methoxyphenyl)-3-trifluoromethyl pyrazole [SC-560]), COX-2 (N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide [NS-398], rofecoxib), or COX-3 (antipyrine), or a non-selective inhibitor of the COX-1/2 isoforms (ketoprofen) did not protect mice from neurotoxicity. Finally, methamphetamine did not change striatal prostaglandin E_2 (PGE_2) content. Taken together, these data suggest that COX-2 is an obligatory factor in methamphetamine-induced neurotoxicity. The functional aspect of COX-2 that contributes to drug-induced neurotoxicity does not appear to be its prostaglandin synthetic capacity. Instead, the peroxidase activity associated with COX-2, which can lead to the formation of reactive oxygen species and dopamine quinones, can account for its role.

Methamphetamine (METH) is an addictive drug of abuse that causes persistent damage in dopamine (DA) nerve endings of the striatum. Signs of METH-induced neurotoxicity include inhibition of tyrosine hydroxylase (Haughey et al., 1999), inactivation of the DA transporter (Metzger et al., 2000), reductions in function of the vesicle monoamine transporter (Baucum et al., 2004), deficits in mitochondrial energy production (Burrows et al., 2000), and apoptosis (Davidson et al., 2001; Cadet et al., 2003). The mechanisms by which METH causes nerve ending damage are not fully understood, but considerable evidence points to oxidative stress as a probable mechanism (Kita et al., 2003). Clues for a new mediator of METH neurotoxicity emerged from a recent microarray analysis of drug-induced changes in gene expression. We found that a neurotoxic regimen of METH increased expression of the genes for transcription factor CCAAT/enhancer-binding protein (C/EBP) and COX-2 (Thomas et al., 2004a). The COX enzymes are the obligatory step in prostaglandin biosynthesis and exist in two major isoforms: COX-1 is constitutively expressed whereas COX-2 expression is inducible (Smith et al., 2000). The coincident induction of the C/EBP and COX-2 genes in the early phases of METH intoxication is significant for several reasons. First, COX-2 and the prostaglandins have been implicated as mediators of neuronal inflammation and damage that accompanies injury and disease of the CNS (Simmons et al., 2004), including that caused by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a highly-selective DA neurotoxin (Feng et al., 2002; Teismann et al., 2003), ischemic brain injury (Iadecola et al., 2001), and glutamate-mediated excitotoxicity (Mirjany et al., 2002). Second, C/EBP exerts transcriptional control over the COX-2 gene (Caivano et al., 2001) and its expression is increased by brain injury (Walton et al., 1998; Cortes-Canteli et al., 2004).

The present studies were designed to test the possibility that COX-2 plays an important role in METH-induced neurotoxicity. We report presently that METH increases the expression of COX-2 protein in striatum, while expression of COX-1 remains unchanged. Genetic inactivation of the COX-2 gene, but not the COX-1 gene, renders mice resistant to METH-induced neurotoxicity. These results establish COX-2 as an obligatory mediator of the DA nerve terminal damage caused by METH.

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METHODS

Materials: (+) methamphetamine hydrochloride, NS-398, ketoprofen, antipyrine, SC-560, DA, methanol, EDTA, and all buffers and HPLC reagents were purchased from Sigma-Aldrich (St. Louis, MO). Rofecoxib was generously provided by Merck Research Laboratories (Rahway, NJ). PGE_2 enzyme immunoassay kits and antibodies against COX-1 (monoclonal) and COX-2 (polyclonal) were obtained from Cayman Chemical (Ann Arbor, MI). Horseradish peroxidase-conjugated secondary antibodies were obtained from Amersham Biosciences (Piscataway, NJ). Bicinchoninic acid protein assay kits were purchased from Pierce (Rockford, IL) and Western Lightning Chemiluminescence Reagent Plus was obtained from Perkin Elmer Life Sciences (Boston, MA). Biomax MR film was from Kodak (Rochester, NY).

Animals: Wild-type, female C57BL/6 mice were obtained from Harlan (Indianapolis, IN). Female mice with null mutations (homozygous) of the COX-1 (strain B6;129P2-*Ptgs1*^{tm1Unc}) or COX-2 gene (strain B6;129P2-*Ptgs2*^{tm1Smi}) and their wild-type littermates were purchased from Taconic Farms (Germantown, NY). The COX-1 and COX-2 knockout mice are bred on a mixed B6;129P2 background using a rotational scheme, as described by the supplier. All mice weighed 20-25 g at the time of experimentation and were housed in a temperature controlled room on a 12 hr alternating light-dark cycle. Mice had free access to food and water. The Institutional Care and Use Committee of Wayne State University approved the animal care and experimental procedures. All procedures were also in compliance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Pharmacological and physiological procedures: All animals were group-housed in acrylic cages with microisolator lids prior to and during experimentation. The ambient temperature within the treatment room was 23.0 ± 0.2°C, and was monitored throughout all experiments. METH was injected i.p. at a dose of 5 mg/kg in 4 injections with a 2 hr interval between each injection. Controls received i.p. injections of physiological saline on the same schedule as METH. Injection volumes were 0.1 ml/10 g body weight. Mice

were also treated with various COX inhibitors to determine their effects on METH-induced neurotoxicity. These drugs and their injection schedules were administered as follows. Rofecoxib (COX-2 inhibitor), was administered by gavage in 0.5% methylcellulose in a dose of 25 mg/kg once per day for 5 days preceding METH treatment. Rofecoxib was also administered 1 hr before the first METH injection, 1 hr after the last METH injection, and once daily thereafter until the time of sacrifice. NS-398 (COX-2 inhibitor) was administered i.p. in a dose of 10 mg/kg in 40% DMSO 1 hr before the first METH injection, 1 hr after the last METH injection, and twice daily thereafter until the time of sacrifice. SC-560 (COX-1 inhibitor) and ketoprofen (COX-1/2 inhibitor) were administered i.p. in doses of 10 mg/kg in 40% DMSO 1 hr before the first METH injection and 1 hr after the last METH injection. Antipyrine (COX-3 inhibitor) was administered i.p. in a dose of 100 mg/kg in physiological saline 30 min prior to each METH injection. Body temperature was monitored throughout all treatments by telemetry using IPTT-200 implantable temperature transponders from Bio Medic Data Systems, Inc. (Seaford, DE). Core body temperatures were recorded non-invasively using the DAS-5001 console system from Bio Medic. These mice were sacrificed 48 hr after the above treatments for neurochemical analyses.

Immunoblotting: Striatal tissue was dissected from brain at the indicated times (below) after METH treatment and stored frozen at –80ºC. Frozen tissue was disrupted by sonication in 1% SDS, 10 mM Tris pH 7.4, 1 mM sodium orthovanadate at 95ºC and insoluble material was sedimented by centrifugation. Protein was determined by the bicinchoninic acid method and equal amounts of protein (100 μg) were resolved by SDS-PAGE and then electroblotted to nitrocellulose. Blots were blocked in Tris-buffered saline containing Tween-20 (0.1% v/v) and 5% non-fat dry milk for 1 hr at room temperature. COX-1 and COX-2 proteins were detected on the same blots by incubating with anti-COX-1 (diluted 1:400) or anti-COX-2 (diluted 1:2000) respectively for 16 hr at 4°C. After exposure to primary antibodies, blots were washed and incubated with the appropriate horseradish peroxidase-conjugated anti-IgG secondary antibody (1:3000, 1 hr at room temperature). Immunoreactive bands were visualized by enhanced chemiluminescence.

Determination of striatal DA and PGE_2 content: Depletion of striatal DA after METH treatment is widely used as an index of METH-induced toxicity to DA nerve endings. DA depletion from striatum faithfully reflects other measures of DA nerve ending damage caused by METH, such as reduced tyrosine hydroxylase immunoreactivity or reduced ligand binding to the DA transporter. Striata were dissected from brain 48 hr after METH treatment and stored frozen at –80ºC. Tissues were weighed and sonicated in 5 vol of 0.1 M perchloric acid at 4°C. Insoluble protein was removed by centrifugation and catecholamines were adsorbed to alumina. After elution from alumina, the amount of DA in samples was determined by HPLC with electrochemical detection by comparison to a standard curve of authentic DA. Striatal PGE_2 content was determined by enzyme immunoassay using a commercially available kit as previously described (Ayoub et al., 2004).

Data analysis: The effects of METH (± COX inhibitors) on striatal DA and PGE_2 content were tested for significance by ANOVA. Individual treatment groups were compared to appropriate controls using Dunnett's Multiple Comparison Test in GraphPad Prism 4. Differences were considered significant if $p < 0.05$. Areas under the curve (AUC) for core body temperature responses over the 9 hr treatment periods were calculated by comparison against a common baseline for all groups using GraphPad Prism 4 and were expressed in arbitrary units.

RESULTS

A neurotoxic regimen of METH increased expression of COX-2 protein in the striatum of wild-type C57BL/6 mice. The immunoblot in Fig. 1A shows that COX-2 was increased by 50% as early as 3 hr after the final METH injection. COX-2 expression continued to increase up to 48 hr after treatment, reaching a peak of 390% of control at 24 hr. The levels of COX-1 protein were not changed by METH over the same time course. These results are in good agreement with our previous finding that COX-2 gene expression is increased by METH while COX-1 gene expression remains unchanged (Thomas et al., 2004a). Using the time point of peak expression (24 hr), we examined COX-2 protein levels in the wild-type littermates of COX-1 and COX-2 knockout mice (Fig. 1B). As with the wild-type C57BL/6 mice, these animals also exhibit robust increases in COX-2 expression of up to 320% of controls at this time point.

To test active involvement of COX-2 in METH-induced neurotoxicity, mice with targeted deletion of the genes for either COX-1 or COX-2, as well as their wild-type littermates, were tested for their responses to drug. Fig. 2 shows that striatal DA content was the same among the five groups of mice under control conditions. METH lowered striatal DA by about 70% in all wild-type mice. COX-1 knockout mice responded to METH in the same fashion as wild-type mice, showing reductions in striatal DA to 35% of control. On the other hand, COX-2 knockout mice were resistant to METH-induced neurotoxicity. DA levels were reduced to 81% of control in COX-2 knockout mice. The effect of METH on striatal DA content was significant in all 5 groups ($p < 0.01$, Dunnett's Multiple Comparison Test), but the DA levels in COX-2 knockouts were significantly higher than all wild-type and COX-1 knockout mice after METH treatment. In agreement with results shown in Fig. 1, METH increased COX-2 expression in COX-1 knockout mice, but did not change COX-1 expression in COX-2 knockouts (data not shown). In view of results showing that C57BL/6 wild-type mice responded to METH in an identical manner as the wild-type littermates of the COX-1 and COX-2 knockout mice, and considering that drug-effects in the COX-2 knockouts were so different from

the responses in COX-1 knockouts, strain-effects on METH actions can be ruled out and C57BL/6 mice are used hereafter as wild-type controls.

One aspect of the COX-2 knockout phenotype is a hampered thermoregulatory response to pyrogens (Li et al., 2001). This is potentially significant because lowered core temperature is neuroprotective against METH (Bowyer et al., 1992; Miller and O'Callaghan, 1994). Fig. 3 shows the core body temperature responses of mice to METH. The core temperatures of all groups of mice under control conditions did not differ significantly, so the results are included in a single control plot to make inspection of the data easier. It can be seen that METH increased body temperature by about 1°C in wild-type mice, starting 1 hr after the first METH injection and lasting for approximately 6 hr. The initial response to METH of both COX-1 and COX-2 knockout mice was a fall in core temperature of 1-2°C below control which lasted for about 2 hr. Thereafter, core temperatures of both groups increased gradually during the METH treatment regimen, and by 4 hr after the start of drug treatment, the COX-1 and COX-2 knockout groups had core temperatures that were the same as METH-treated wild-type mice. Core temperatures of all groups returned to control levels by 1-2 hr after the last METH injection as shown in Fig. 3. AUCs were also calculated for each group to provide a measure of the overall response to METH throughout the entire treatment period (-60 to 480 min). These measures confirm the data in Fig. 3 and indicate that wild-type mice treated with METH (AUC=2514) showed the largest total response by comparison to controls (AUC=2160). The lesser responses of the COX-1 (AUC=2232) and COX-2 (AUC=2142) knockout mice to METH can be accounted for by their initial 120 min period of hypothermia after drug treatment.

In view of results showing that genetic inactivation of COX-2 was protective against METH-induced neurotoxicity, we tested various pharmacological inhibitors of the COX enzymes for neuroprotection in wild-type mice. All doses and treatment schedules used for the COX inhibitors were based on published, confirmed accounts of COX inhibition (see Methods above). Fig. 4 shows that the selective COX-2 inhibitors NS-398 and rofecoxib did not prevent drug-induced reductions in DA. SC-560, a selective COX-1

inhibitor, also failed to protect mice from METH-induced neurotoxicity. The non-selective COX-1/2 inhibitor ketoprofen and the COX-3 inhibitor antipyrine were also ineffective in preventing the effects of METH on DA nerve terminals as shown in Fig. 4. All treatment groups were significantly different from controls ($p < 0.01$, Dunnett's Multiple Comparison test) and none of the groups treated with a COX inhibitor + METH was different from METH alone. None of the COX inhibitors altered striatal DA content when administered without METH (data not shown).

The effects of the COX inhibitors on METH-induced hyperthermia are shown in Fig. 5. When given alone, NS-398 caused a drop in body temperature of 1-2°C that started almost immediately after injection and lasted for 2-3 hr (data not shown). Fig. 5A presents results in mice treated with METH plus COX-2 inhibitors. The hyperthermic response to METH was very similar to what was observed in Fig. 3. However, core body temperature responses to METH were quite different in the NS-398 and rofecoxib groups. Mice treated with NS-398 responded with a further drop in body temperature of about 2°C that developed rapidly after initiation of METH treatment and remained below control for 3 hr. On the other hand, mice treated with rofecoxib developed a slightly greater hyperthermia than mice treated with METH alone. This effect of rofecoxib persisted through 3 METH injections and returned to control levels thereafter. The overall effects of the COX-2 inhibitors were reflected in AUCs and confirmed that METH (AUC=2562) and METH + rofecoxib (AUC= 2628) treated mice were hyperthermic by comparison to controls (AUC= 2208), whereas the METH + NS-398 group was hypothermic (AUC= 2133).

The effects of SC-560, ketoprofen, and antipyrine in METH treated mice are presented in Fig. 5B. By themselves, each COX inhibitor lowered body temperature by 1-2°C for about 2-3 hr (data not shown). As seen with the COX-2 inhibitors (Fig. 5A), the responses to these COX inhibitors were quite varied. SC-560, a COX-1 inhibitor, caused a profound hypothermia immediately after the start of METH treatment, with core temperatures lowered by almost 2°C for 1 hr. The SC-560 + METH treated group remained at control core temperature levels for the next 2 hr and then converted to a

hyperthermia of 2-3°C above controls. This group remained above control temperatures for the remainder of the experiment. Ketoprofen, a non-selective COX-1/2 inhibitor, caused effects on core temperatures that were very similar to SC-560, producing a drop of about 2°C below controls that lasted for 2-3 hr. Thereafter, the ketoprofen + METH group remained near the temperatures of the METH group. Antipyrine initially enhanced METH-induced hyperthermia by about 0.5°C, after which body temperatures returned to levels between controls and METH-treated mice for the remainder of the experiment. Calculation of AUCs for each group confirmed the METH-induced hyperthermia (AUC=2766) by comparison to controls (AUC=2202). AUCs for groups treated with SC-560 (AUC=2514), ketoprofen (AUC=2385), and antipyrine (AUC=2526) revealed an overall hyperthemia throughout the treatment period, as all groups were between controls and METH-treated mice.

In view of results showing that METH increased expression of COX-2 protein (Fig. 1 above), the effects of drug treatment on striatal PGE_2 content was determined. Fig. 6 shows that the levels of PGE_2 remained relatively unchanged for up to 48 hr after METH treatment. There was a slight tendency of a reduction in PGE_2 levels 12-24 hr after treatment, but this effect was not statistically significant.

DISCUSSION

Evidence linking the COX enzymes and prostaglandins to METH-induced neurotoxicity is very limited in scope. Kita and colleagues showed that METH increased COX-2 expression (Kita et al., 2000), and ketoprofen has been shown to provide partial protection against METH-induced neurotoxicity (Asanuma et al., 2003). This situation is somewhat surprising in view of the larger role of the prostanoids in mediating other forms of neuronal injury, inflammation, and degeneration (Hurley et al., 2002). A comprehensive analysis of gene expression changes provoked by a neurotoxic regimen of METH substantiated these earlier findings by showing a drug-induced increase in expression of the genes for COX-2 and C/EBP (Thomas et al., 2004a). These initial gene-expression results are extended presently by the demonstration that METH intoxication induces the expression of COX-2 protein. COX-1 protein expression was not changed in keeping with our finding that METH does not induce the gene for COX-1 (Thomas et al., 2004a). The importance of COX-2 in METH neurotoxicity was further substantiated by the finding that genetic inactivation of the COX-2 gene rendered mice almost totally resistant to drug-induced nerve terminal damage. COX-1 knockout mice were no different from wild-type mice in their response to METH, manifested as significant reductions (about 70%) in striatal DA content. COX-2 expression was increased by METH in the COX-1 knockout mice to the same extent observed in wild-type mice treated with METH (data not shown).

One element of the COX-2 knockout phenotype with possible relevance to METH neurotoxicity is an altered thermoregulatory response. COX-2 knockout mice have a diminished febrile response to pyrogens (Li et al., 2001). Treatments that prevent METH-induced hyperthermia, such as MK-801 or lowered ambient temperature (Bowyer et al., 1994; Miller and O'Callaghan, 1994), also prevent its associated neurotoxicity. Therefore, we studied the core temperature response of COX-2 knockout mice to METH. The initial response of COX-2 knockouts to METH treatment was a rapid fall in body temperature of about 2°C. The response of COX-1 knockouts was the same as COX-2 knockouts whereas wild-type mice showed the expected hyperthermia. This drug-induced

hypothermia in the COX knockout mice lasted for 1-2 hours and returned gradually to levels shown by METH treated wild-type mice. It is significant that both COX-1 and COX-2 knockout mice showed the same core temperature response to METH while only COX-2 knockouts were resistant to its neurotoxicity. It is also interesting that COX-1 mice demonstrated the same extent of DA depletion as wild-type mice, yet COX-1 knockouts responded to METH with hypothermia while wild-type mice developed hyperthermia. Taken together, these data establish that the body temperature responses of COX knockout mice to METH are not predictive of neurotoxicity, and suggest that an altered thermoregulatory mechanism in COX-2 knockout mice is not mediating their resistance to METH-induced neurotoxicity.

The effects of pharmacological inactivation of COX-2 in wild-type mice apparently did not agree with the results seen in mice lacking COX-2. NS-398 and rofecoxib, two relatively selective COX-2 inhibitors, did not provide any measure of protection against the neurotoxic effects of METH. To test the possibility that COX-1 was playing a subtle role that was not immediately apparent, even in the COX-2 knockout mice, the COX-1 selective inhibitor SC-560 was given to mice before METH. It too failed to prevent METH toxicity. Ketoprofen, a non-selective inhibitor of both COX-1/2 isoforms did not protect mice from METH-induced neurotoxicity. A new splice variant of COX-1, referred to as COX-3 (Chandrasekharan et al., 2002), participates in the regulation of body temperature in mice via its constitutive production of prostaglandins (Ayoub et al., 2004), so we tested an inhibitor of this enzyme as well to rule out a possible role for it in METH toxicity. Antipyrine, like other COX inhibitors used presently, did not prevent METH-induced neurotoxicity. The doses and treatment regimens for each COX inhibitor used were based on published, confirmed accounts of COX inhibition in mice, so tests of COX inhibition were not further confirmed presently. Therefore, it is possible that COX-2 was not inhibited by either NS-398 or rofecoxib. This does not seem likely in view of the body temperature responses of mice treated with METH + COX-2 inhibitors (Fig. 5A), suggesting that NS-398 and rofecoxib were pharmacologically active. The inhibitors of COX-1, COX-3, and COX-1/2 produced widely divergent effects on METH-induced hyperthermia (Fig. 5B), suggesting that these

drugs were also given in pharmacologically active doses. Finally, the COX inhibitors caused mild hypothermia by comparison to controls when given without METH, suggesting effective COX inhibition (Ayoub et al., 2004). It is not immediately apparent why ketoprofen was ineffective against METH in our studies while it partially reduced METH toxicity when tested by Asanuma and colleagues (Asanuma et al., 2003). These investigators used lower doses of METH (4 mg/kg versus 5 mg/kg) and a slightly different ketoprofen treatment regimen (2 or 4 mg/kg before each of 4 METH injections) than used presently, but the effect of ketoprofen on body temperature seen by Asanuma et al. (2003) after METH administration was identical to our findings of an initial hypothermia that transitioned to a hyperthermia. COX inhibitors are not uniformly neuroprotective and their effects on MPTP-induced damage to the DA neuronal system is unpredictable. Some COX inhibitors protect against MPTP (Teismann and Ferger, 2001; Maharaj et al., 2004) while others do not (Sairam et al., 2003). The reasons for widely varying effects of COX inhibitors on drug-induced neural damage are not clear.

The apparent differences in outcome between genetic inactivation of COX-2 (i.e., protective) versus pharmacological inhibition of the enzyme (i.e., COX-2 inhibitors not protective) with regard to METH-induced neurotoxicity could be explained if COX-2 was mediating a process that was independent of its prostaglandin biosynthetic capacity. One clue for the operation of such a mechanism emerged from studies showing that PGE_2 levels were not increased in striatum after METH intoxication (Fig. 6), despite the fact that COX-2 protein levels were elevated. It is well-known that the COX enzymes express peroxidase enzymatic activity in addition to the cyclooxygenase function (Smith et al., 2000). This is highly significant with respect to METH-induced neurotoxicity because the peroxidase activity associated with COX-2 converts DA to its quinone (Hastings, 1995). DA quinones are elevated in striatum by METH (LaVoie and Hastings, 1999) and they have also been implicated recently in MPTP-induced degeneration of DA neurons (Teismann et al., 2003). The quinones of DA can modify numerous proteins whose function is altered by the neurotoxic amphetamines including tryptophan hydroxylase (Kuhn and Arthur, 1998), tyrosine hydroxylase (Kuhn et al., 1999), and the DA transporter (Whitehead et al., 2001). The covalent modification of proteins by catechol

quinones can generate additional reactive species and radicals through creation of redox-cycling centers/moieties (Paz et al., 1991).

One of the primary pharmacological effects exerted by the neurotoxic amphetamines, including METH, is a release of DA into the cytoplasm from synaptic vesicles and eventually into the synaptic space via reverse transport through the DA transporter (Sulzer et al., 1995). This liberation of DA from its presynaptic storage sites, along with increased production of COX-2 protein, would create conditions that favor the production of catechol quinones. In addition to their direct effects on proteins, membrane-bound DA quinones (Le et al., 2001) and free DA quinones (manuscript in preparation) cause extensive microglial activation. Recently emerging data has implicated microglial activation as an early event in the neurotoxic cascade that is initiated by the neurotoxic amphetamines (LaVoie et al., 2004; Orio et al., 2004; Thomas et al., 2004b). Therefore, the requirement for COX-2 as an essential factor in at least METH-induced neurotoxicity offers a potential mechanism by which this drug of abuse stimulates the production of DA quinones and leads to microglial activation. This possibility is currently under investigation.

A major clinical danger associated with high-dose METH abuse in humans is the development of hyperthermia. The neurotoxic effects of METH can be blocked by drugs or treatments that prevent hyperthermia (Bowyer et al., 1994; Miller and O'Callaghan, 1994), making it difficult to determine if hyperthermia is a direct participant in drug-induced neurotoxicity, or a response that is coincident but does not actually contribute to nerve terminal damage. Several aspects of our data support the latter contention. First, COX-1 and COX-2 knockout mice respond in an identical manner to METH with a rapidly developing hypothermia, yet COX-1 mice show the same toxicity as wild-type mice, and COX-2 knockouts show very little DA nerve terminal damage. Second, the COX-2 inhibitors did not prevent METH-induced neurotoxicity but when administered with METH, NS-398 caused a significant hypothermia and rofecoxib enhanced hyperthermia. Third, inhibitors of COX-1, COX-3, or COX-1/2, when administered along with METH, led to varying body temperature responses, including hypothermia that

transitioned over time into hyperthermia (see Fig. 5B). Taken together, these results with COX knockout mice and COX enzyme inhibitors indicate that core temperature responses to METH (i.e., hypothermia or hyperthermia) cannot predict the subsequent neurochemical response (i.e., toxicity or neuroprotection) to drug administration, and lead us to agree with the conclusion that hyperthermia is neither necessary nor sufficient for amphetamine-induced neurotoxicity (Ali and Itzhak, 1998; Darvesh and Gudelsky, 2004).

Acknowledgements

We thank Drs. David DeWitt (Michigan State University), Daniel Simmons (Brigham Young University) and Robert Langenbach (NIH, NIEHS) for their helpful comments and expert advice on COX-2 involvement in METH actions. We also thank Merck Research Labs for the generous gift of rofecoxib.

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Footnotes

This research was supported by National Institutes of Health Grants DA10756 and DA014392, and a VA Merit Award.

Legends for Figures

Fig. 1. Effects of METH on COX protein expression. (A) Wild-type C57BL/6 mice were treated with a neurotoxic regimen of METH and sacrificed at the indicated times. Striatal expression of COX-2 and COX-1 was determined by immunoblotting as described in Methods. Results are representative of 5 independent experiments. (B) Wild-type C57BL/6 mice and the wild-type littermates of COX-1 and COX-2 knockout mice were treated with physiological saline (Control; n=3) or a neurotoxic METH regimen (METH; n=5) and sacrificed at 24 hours to examine COX-2 protein expression.

Fig. 2. Effects of METH on striatal DA content in COX knockout mice. Wild-type C57BL/6 (C57 WT), wild-type COX-1 (COX-1 WT) and COX-2 (COX-2 WT) knockout (KO) littermates, COX-1 KO mice, and COX-2 KO mice were treated with physiological saline (filled columns) or a neurotoxic regimen of METH (open columns) and sacrificed 48 hr later. Striatal tissue was analyzed for DA content by HPLC with electrochemical detection. Data are presented as ng of DA per milligram of tissue (wet weight) and are the means ± SEM (n= 8-10 mice/group). The overall effect of treatment was significant at $p < 0.01$ by ANOVA. The effect of METH was significantly different from control in all groups (*, $p < 0.01$, Dunnett's Multiple Comparison test) and METH-treated COX-2 KO mice were significantly different from all other METH-treated mice (**, $p < 0.01$, Dunnett's Multiple Comparison test).

Fig. 3. Effects of METH on core body temperature in COX KO mice. Wild-type mice (●), COX-1 KO (□), or COX-2 KO (■) mice were treated with a neurotoxic regimen of METH as described in Methods. Controls (○) received physiological saline according to the same injection schedule used for METH. Core body temperatures of mice were recorded by telemetry for 60 min before METH injection and for 480 minutes thereafter. METH injections are indicated by vertical arrows on the x-axis. Data are presented as mean core body temperature (°C) for each group (n=4-5 mice/group) at the indicated times. SEM bars are omitted for the sake of clarity and were less than 10% of the mean in all groups.

Fig. 4. Effects of COX inhibitors on METH-induced neurotoxicity. Mice were treated with physiological saline (control) or a neurotoxic regimen of METH. Where indicated, mice treated with METH also received injections of COX-2 inhibitors (NS-398 and rofecoxib), a COX-1 inhibitor (SC-560), a nonselective inhibitor of COX-1/2 (ketoprofen), or an inhibitor of COX-3 (antipyrine). Doses and injection schedules used for the COX inhibitors are described in Methods. Mice were sacrificed 48 hr after treatment and striatal DA content was determined by HPLC with electrochemical detection. Data are presented as % control and represent means ± SEM of groups that contained 4-6 mice/group. The effects of METH alone and METH + each COX inhibitor were signficantly different from control (*, $p < 0.01$, Dunnett's Multiple Comparison test).

Fig. 5. Effects of COX inhibitors on core body temperature responses to METH. (A) Mice (n=4-6/group) were treated with physiological saline (○), METH (●), METH + NS-398 (■), or METH + rofecoxib (□). In panel (B), mice were treated with physiological saline (○), METH (●), METH + SC-560 (□), METH + ketoprofen (■), or METH + antipyrine (◊). Core body temperatures of mice were recorded by telemetry for 60 min before METH injection and for 480 minutes thereafter. METH injections are indicated by vertical arrows on the x-axis. Data are presented as mean core body temperature (°C) for each group (n=4-5 mice/group) at the indicated times. SEM bars are omitted for the sake of clarity and were less than 10% of the mean in all groups.

Fig. 6. Effects of METH on striatal PGE_2 levels. Mice were treated with a neurotoxic regimen of METH and sacrificed at the indicated times. Striatal tissue was analyzed for PGE_2 content by enzyme immunoassay. Data are presented as pg PGE_2 per milligram of tissue (wet weight) and represent mean ± SEM of groups containing 4-5 mice per treatment time.

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Figure 1

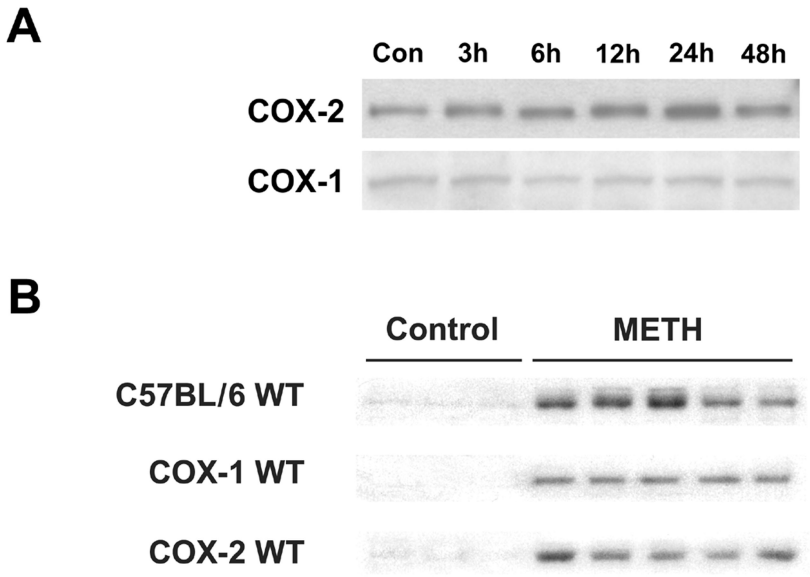

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Figure 2

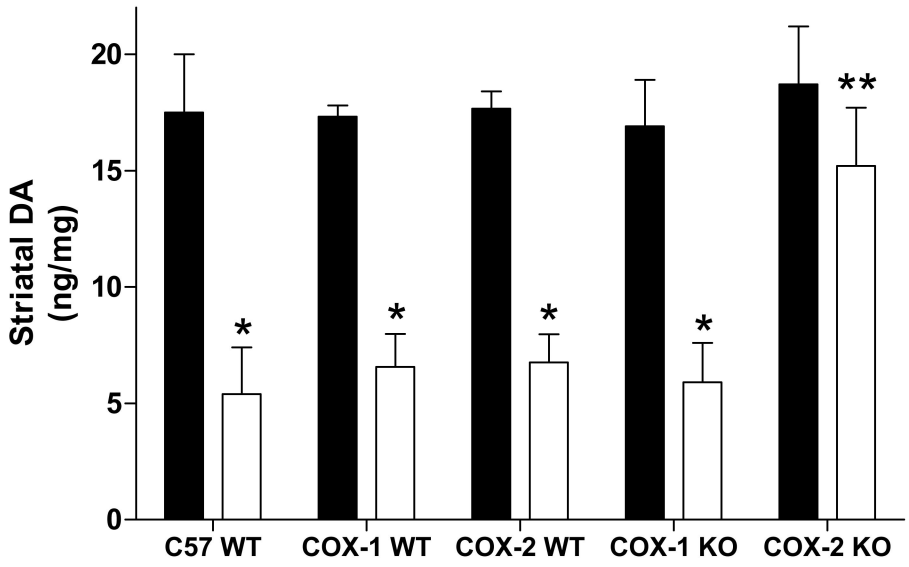

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Figure 3

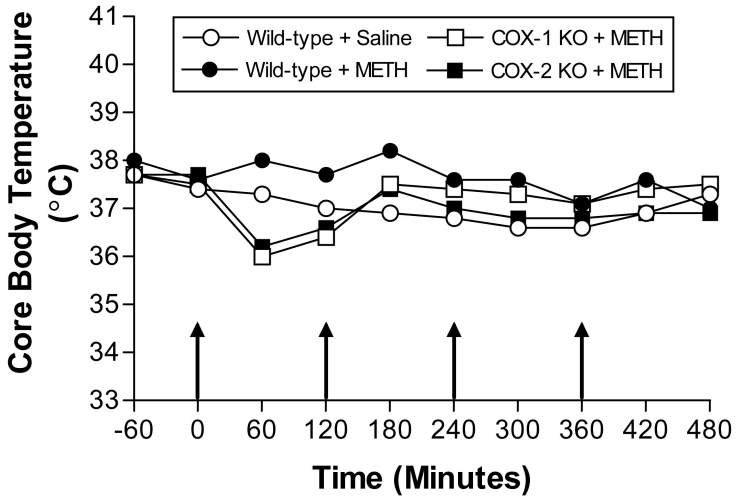

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Figure 4

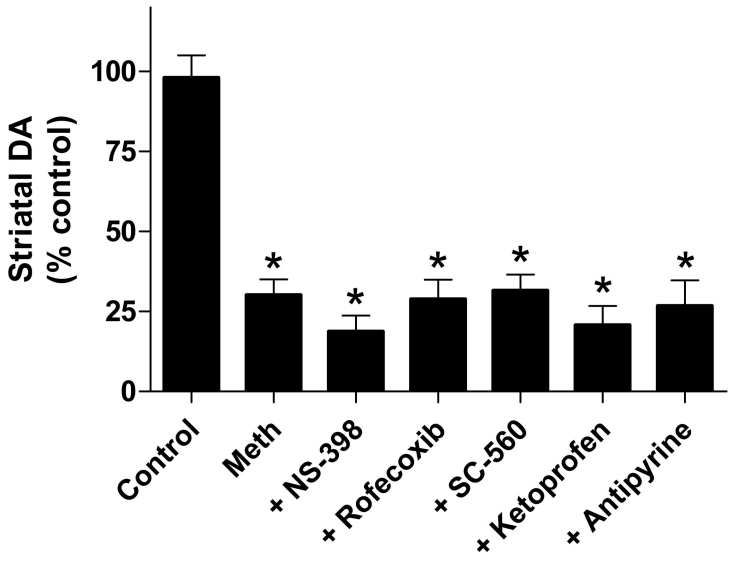

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Figure 5

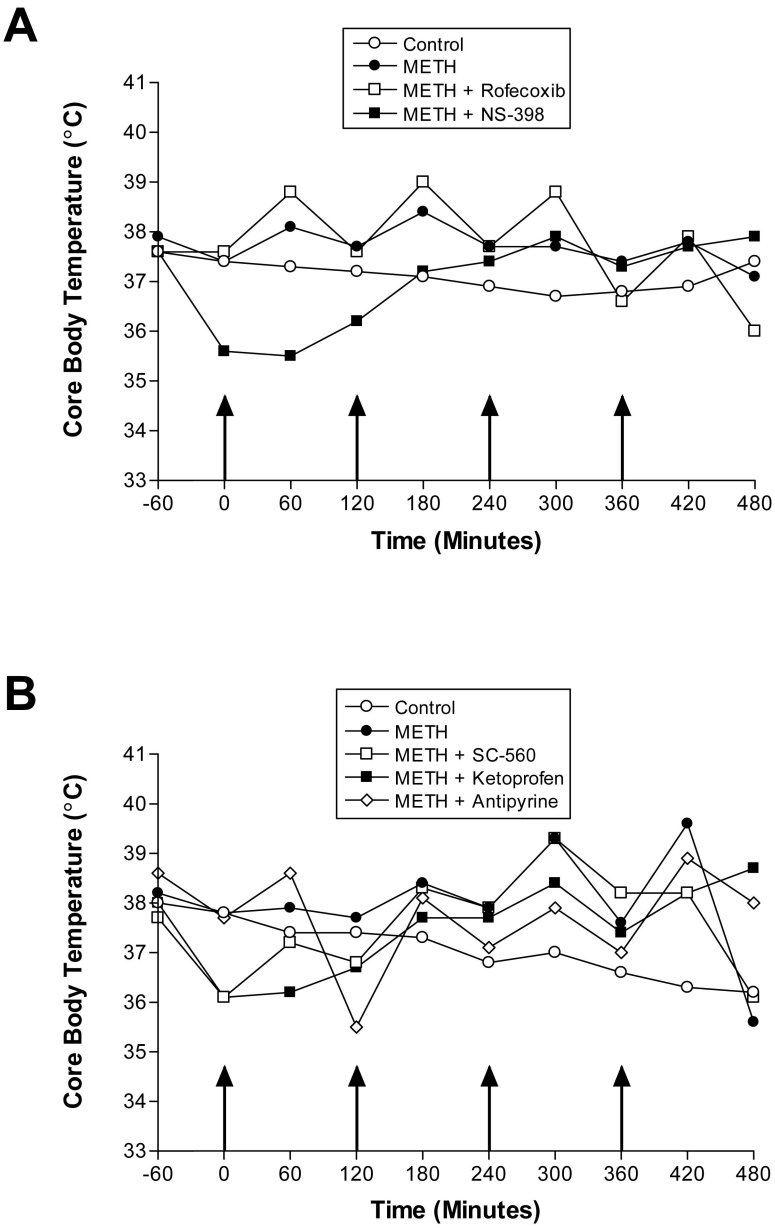

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Figure 6