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Ascorbic Acid Inhibits Spinal Meningeal Catechol-o-Methyl Transferase in Vitro, *Markedly Increasing Epinephrine Bioavailability*

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Background: **The spinal meninges have previously been shown to contain catechol-o-methyl transferase (COMT), the enzyme that metabolizes epinephrine to the inactive metabolite metanephrine. The authors of this study aimed to quantitate the metabolism of epinephrine traversing the spinal meninges and to determine if that metabolism could be inhibited. In addition, they tried to determine the meningeal permeability of epinephrine.**

Methods: ***Macca nemestrina*** **spinal meninges were mounted in a diffusion cell and epinephrine was added to the donor reservoir at time 0. Three hundred minutes later, all buffer in the recipient reservoir was collected and analyzed for epinephrine metabolites. The experiments were conducted with either ascorbic acid (1 mM) or sodium metabisulfite (5.3 mM) added as antioxidants.**

Results: **In the presence of sodium metabisulfite, 60 ± 6% of the epinephrine traversing the meningeal specimens was metabolized by COMT. In contrast, in the presence of ascorbic acid, less than 3% of the epinephrine traversing the spinal meninges was metabolized by COMT ($P = 0.0001$). The meningeal permeability coefficient for epinephrine was 0.38 ± 0.08 cm/min × 10^{-3}.**

Conclusions: **Epinephrine permeability through the spinal meninges is low, and meningeal COMT markedly reduces the bioavailability of what little epinephrine can traverse the meninges. However, a clinically relevant concentration of ascorbic acid, a competitive inhibitor of COMT, almost completely blocks epinephrine metabolism and increases the bioavailability of epinephrine. (Key words: Ascorbic acid. Epinephrine, diffusion. Metabolism, metanephrine. Spinal meninges, monkey.)**

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EPINEPHRINE is commonly added to epidurally administered drugs and has been shown to prolong the duration and improve the quality of both epidural local anesthetic block and epidural opioid analgesia.[1–7] However, it is not known if this beneficial effect of epinephrine results from a direct analgesic effect of epinephrine within the spinal cord dorsal horn or from reduced drug clearance from the epidural space caused by epinephrine-induced vasoconstriction.

Previously we showed that epinephrine is metabolized within the spinal meninges by catechol-o-methyl transferase (COMT).[8] This investigation suggested that the spinal meninges may limit the bioavailability of epinephrine at analgesic sites within the spinal cord dorsal horn. However, the methodology used in this earlier study did not allow us to determine what percentage of the epinephrine traversing the spinal meninges was actually metabolized. Thus one of our goals was to quantitate the metabolism of epinephrine traversing the spinal meninges.

Given that epinephrine improves epidural anesthesia and analgesia, increasing the bioavailability of epidurally administered epinephrine may further improve the efficacy of epidurally administered local anesthetics and opioids. Thus a second goal of this study was to determine if epinephrine metabolism by meningeal COMT could be inhibited.

To address these goals we used a previously validated diffusion cell model[9–11] to quantitate the flux of epinephrine and its metabolites through *macca nemestrina* spinal meninges. In addition, we investigated the ability of ascorbic acid, an inhibitor of COMT in some systems,[12] to reduce epinephrine metabolism.

Materials and Methods

Studies were approved by the University of Washington Animal Care and Use Committee, and guidelines of

the American Association for Accreditation of Laboratory Animal Care were followed throughout.

Tissue Sources

Tissue was obtained from animals (*M. nemestrina*) scheduled to be killed as part of the tissue distribution program of the University of Washington Regional Primate Research Center. All meningeal specimens were removed from animals anesthetized with thiopental and ketamine. Animals of either sex weighing an average of 8.6 ± 0.5 kg were used.

Tissue Preparation

The spinal cord was exposed from T5 to L5 by laminectomy. The spinal cord was removed *en bloc* and all three meningeal layers were carefully reflected from the spinal cord, preserving their normal anatomic relations. Intact meningeal specimens measuring approximately 4 cm^2 were placed between two halves of a temperature-controlled diffusion cell with a 0.785-cm^2 port connecting the donor and recipient reservoirs. Ten milliliters of mock cerebrospinal fluid (CSF) (140 mEq NaCl, 25 mEq $NaHCO_3$, 2.9 mEq KCL, 0.4 mEq $MgCl_2$, 3.5 mEq urea, 4 mEq glucose, and 2 mEq $CaCl_2$; *p*H = 7.38–7.42; 292–298 mOsm) were placed in the fluid reservoirs on either side of the meningeal tissue. Air and carbon dioxide (5%) were bubbled through each fluid reservoir to maintain normal *p*H and provide oxygen to the meningeal cells. In addition, because epinephrine can be oxidized spontaneously in air, either ascorbic acid (1 mM, n = 10) or sodium metabisulfite (5.3 mM, n = 8) was added to the donor reservoir and the recipient reservoir to prevent oxidation. Metabisulfite was chosen as an antioxidant because it is the antioxidant present in commercially available vials of epinephrine and in local anesthetics with previously added epinephrine. Ascorbic acid was chosen as an antioxidant because it is also present in some local anesthetics with previously added epinephrine and because of evidence that it inhibits COMT in some systems.

Epinephrine Metabolism

After allowing at least 20 min for the chambers to equilibrate to 37°C, either 50 μg (metabisulfite experiments), 100 μg (n = 5 ascorbic acid experiments), or 200 μg (n = 5 ascorbic acid experiments) of epinephrine base was added to the donor reservoir on the dura mater side of the diffusion cell. After 300 min, all 10 ml of the mock CSF from the recipient reservoir was collected and acidified with 1.5 ml of 0.1 M perchloric acid. The samples were frozen at −20°C until analyzed for epinephrine, norepinephrine, metanephrine, normetanephrine, and 3-methoxytyramine. Because some metabolites were present in very low concentrations, it was necessary to allow the experiment to proceed for 300 min so metabolite concentrations could be measured accurately.

Epinephrine Permeability

The meningeal permeability coefficient for epinephrine was determined as previously described.[9–11,13] Briefly, diffusion cells were set up as described above and ascorbic acid (0.5 mM) was added to the donor and recipient reservoirs to prevent metabolism and spontaneous oxidation of epinephrine.[14] At time 0, 100 μg epinephrine base (55 μM) and 0.6 μCi ^{3}H-epinephrine (^{3}H-epinephrine; specific activity, 80.7 Ci/mmol; radiochemical purity, 97.1%; NEN® Research Products, DuPont Co., Boston, MA) were added to the donor reservoir. Thereafter, at 10-min intervals for 100 min, 200-μl samples were removed from the donor and the recipient reservoirs and placed in borosilicate scintillation vials for later determination of epinephrine concentration.

The epinephrine concentration in the recipient reservoir *versus* time data were plotted, and epinephrine flux (Q) was determined as the slope of the regression line through these data points. The permeability coefficient for epinephrine was then calculated from the experimentally determined flux using the equation

$$P = Q/C_1 \cdot A$$

where P = permeability coefficient, Q = experimentally determined epinephrine flux, C_1 = initial epinephrine concentration in the donor reservoir, and A = cross-sectional area of meninges available for diffusion (*i.e.*, area of the port connecting the two halves of the diffusion cell).

Chemical Analysis

Epinephrine metabolite concentrations were determined using a Bio-Rad Laboratories modification of the method of Jouve *et al.*[15] (Bio-Rad instruction manual no. 195-6606, Bio-Rad Laboratories Clinical Division, Hercules, CA). Briefly, 4-O-methyldopamine was added as an internal standard and the samples were separated by high-performance liquid chromatography using isocratic separation (Waters 590 Pump and Waters 745 B Data Module; Millipore, Milford, MA). Metabolites were

detected by an electrochemical detector (Coulochem model 5100 A and model 5011 analytical cell; ESA Inc., Bedford, MA). The limits of quantification for metanephrine, normetanephrine, and 3-methoxytyramine were 10 ng/ml with a coefficient of variation of 10% at a concentration of 100 ng/ml and 7% at a concentration of 500 ng/ml.

Epinephrine and norepinephrine concentrations in the epinephrine metabolism experiments were determined in a similar manner except that dehydroxy-benzolamine was used as the internal standard.

Epinephrine concentration in the permeability experiments was determined using radiotracer methods because the sample size and quantity of epinephrine present were too small for other analytical methods. Hydrofluor™ (National Diagnostics, Atlanta, GA) scintillation fluid (5–10 ml) was added to each sample and the samples were counted in a liquid scintillation counter (Tricarb 2000; Packard, Downers Grove, IL) for 10 min or until the standard deviation of depurations per minute was 2% or less. Background radioactivity was determined by counting mock CSF without added radiotracer and was subtracted from the depurations per minute of each sample.

Statistical Analysis

Differences between antioxidants in the amount of epinephrine and metabolites measured in the recipient reservoir were assessed for statistical significance using Student's t test. $P < 0.05$ was considered significant. Data are presented as the mean ± SE.

Results

Metabisulfite

With metabisulfite as the antioxidant and an initial epinephrine concentration of 22.5 nM (1:200,000), the epinephrine concentration in the recipient reservoir after 300 min averaged 120 ± 52 pmol/ml. The concentration of epinephrine metabolites (metanephrine and normetanephrine) averaged 153 ± 55 pmol/ml. Thus metabolites represented an average of 60 ± 6% of the catechols entering the meninges and traversing to the donor reservoir. The methylated metabolite of epinephrine, metanephrine, was produced by the meningeal tissues of all eight animals studied and averaged 99.5 ± 43 pmol/ml. Metanephrine was further metabolized to normetanephrine in two of the animals. Normetanephrine was present at an average concentration of 216 ± 44 pmol/ml. The average metanephrine concentration in meningeal tissues from these two animals was 56 ± 21 pmol/ml, which did not differ significantly from the average metanephrine concentration in the remaining six animals (114 ± 48 pmol/ml).

Ascorbic Acid

Use of ascorbic acid as the antioxidant significantly reduced the production of epinephrine metabolites. At an initial epinephrine concentration of 55 nmol/ml (*i.e.*, 1:100,000) in the donor reservoir, the epinephrine concentration in the recipient reservoir after 300 min averaged 2,480 ± 973 pmol/ml. The concentration of epinephrine metabolites averaged 82 ± 25 pmol/ml or 2.9 ± 0.6% of the catechols entering the spinal meninges and traversing to the donor reservoir. Thus the percentage of epinephrine metabolism was markedly less when ascorbic acid was used as the antioxidant compared with metabisulfite ($P = 0.0001$). Metanephrine was the only epinephrine metabolite detected.

At an initial epinephrine concentration of 110 nmol/ml (*i.e.*, 1:50,000) in the donor reservoir, the epinephrine concentration in the recipient reservoir after 300 min averaged 3,310 ± 383 pmol/ml. The concentration of epinephrine metabolites (metanephrine only) averaged 92 ± 12 pmol/ml or 2.7 ± 3% of the catechols entering the spinal meninges and traversing to the donor reservoir. Again, the percentage of metabolites was markedly less than that produced when metabisulfite was used as the antioxidant ($P = 0.0001$). Metanephrine was the only epinephrine metabolite detected.

Permeability Coefficient

The correlation coefficient (r) for each regression line used to determine epinephrine flux through the tissues being studied was in each case greater than or equal to 0.97, indicating excellent fit of the data to a linear model. The permeability coefficient for epinephrine was 0.38 ± 0.08 cm/min × 10^{-3}.

Discussion

In an earlier study we demonstrated the functional presence of COMT in the spinal meninges of monkeys and pigs.[8] Although the results from this earlier study were of interest, the methodology did not allow us to determine whether the amount of epinephrine metabolized by the spinal meninges was sufficient to reduce the spinal availability of epidurally administered epi-

nephrine. The results of the current *in vitro* study clearly suggest that epinephrine metabolism by the spinal meninges markedly reduces the amount of epidurally administered epinephrine that reaches the spinal cord. In fact, the concentration of metabolite measured in the recipient reservoir probably underestimates the amount of metabolite produced by one half. As metabolite is produced in the spinal meninges, it is just as likely to diffuse back into the donor reservoir as it is to diffuse into the recipient reservoir. Therefore, the percentage of epinephrine that is metabolized after entering the spinal meninges may actually be as great as 75% when metabisulfite is used as the antioxidant.

We chose metabisulfite as an antioxidant for investigation because it is used commercially to preserve epinephrine packaged in individual vials and is added to local anesthetics that come with epinephrine already present. The metabisulfite concentration used (5.3 mM) was within the range present in commercial preparations (2.7–8.3 mM). In addition, the epinephrine concentration studied in conjunction with metabisulfite is probably the most common concentration used clinically in the epidural space (1:200,000). Thus, although the epidural space is admittedly more complex than our *in vitro* model, we think the study conditions are clinically relevant with respect to the issue of epinephrine metabolism by the spinal meninges.

Our studies with ascorbic acid present as the antioxidant clearly show that ascorbic acid can inhibit meningeal COMT. Like catechols, the five-membered ring of ascorbic acid contains an enediol. Because COMT methylates the hydroxyl group of enediols, ascorbic acid acts as a competitive inhibitor of the COMT catalyzed methylation of epinephrine. However, ascorbic acid has been characterized as a weak COMT inhibitor with a k_m between 1 μM[12] and 14 mM.[16] Thus ascorbic acid would not be expected to have a clinically important effect on COMT-catalyzed methylation of epinephrine unless it is present at much higher concentrations than epinephrine. When used as an antioxidant in commercially prepared local anesthetics containing epinephrine (such as Marcaine hydrochloride, Sanofi Winthrop Pharmaceuticals, New York, NY), the concentration of ascorbic acid (11 mM) exceeds that of epinephrine (22.5 nM) by a factor of 5×10^5. In the current study, a 9×10^3-fold greater concentration of ascorbic acid reduced the COMT catalyzed methylation of epinephrine to less than 3%. Thus the ascorbic acid concentration used in some commercial epinephrine-containing local anesthetics is probably sufficient to almost completely inhibit meningeal COMT.

As noted previously, epinephrine prolongs the duration and increases the intensity of anesthesia and analgesia that result from some epidurally administered local anesthetics and opioids. The mechanism by which epinephrine produces these effects is unclear. However, our finding of a low meningeal permeability of epinephrine and the high percentage of metabolism within the meninges would suggest that the anesthesia- and analgesia-enhancing effects of epinephrine are more likely the result of actions within the epidural space (*e.g.*, vasoconstriction and resultant reduced drug clearance into the systemic circulation) and not the result of actions within the spinal cord dorsal horn. However, the permeability of epinephrine is not so low as to preclude a spinal site of action if meningeal metabolism of epinephrine were inhibited (*e.g.*, by adding ascorbic acid). For example, the meningeal permeability coefficient for epinephrine (0.38 ± 0.08 cm · s−1 · cm^2) is more than 60% of the rate for morphine (0.62 ± 0.09 cm · s−1 · cm^2),[13] a drug that we know crosses the spinal meninges at sufficient rate and quantity to produce spinal analgesia. Thus the low meningeal permeability of epinephrine would not be expected to prevent epinephrine from reaching the spinal cord in clinically relevant concentrations if epinephrine metabolism were inhibited by ascorbic acid.

As with epidural anesthesia, adding epinephrine to intrathecal local anesthetics has also been shown to improve the quality and prolong the duration of spinal anesthesia.[17,18] The mechanism by which this occurs is not entirely clear, but it is noteworthy that intrathecal adrenergic agonists are potent analgesics in their own right.[19] Thus the effect of intrathecal epinephrine may be, at least in part, the result of a synergistic pharmacodynamic effect. Consequently, inhibition of meningeal COMT might increase the efficacy of intrathecally administered epinephrine by increasing its spinal bioavailability.

Although the current study indicates that the meningeal metabolism of epinephrine is considerable, and that this metabolism can be prevented by adding clinically relevant concentrations of ascorbic acid, it does not indicate whether inhibition of COMT will provide a clinical benefit. However, it is reasonable to assume that the demonstrated beneficial effects of epidurally and intrathecally administered epinephrine will be increased by procedures that increase epinephrine's local

bioavailability. Definitive proof of this hypothesis awaits appropriate *in vivo* human or animal studies.

Given the high aqueous solubility of epinephrine (log octanol: buffer [*p*H = 7.4] distribution coefficient = −2.59), the relatively low meningeal permeability coefficient for epinephrine is consistent with our earlier demonstration that meningeal permeability decreases at the extremes of aqueous solubility.[13]

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