Growth and Angiogenesis Are Inhibited in Vivo in Developing Tissues by Pyrazine and Its Derivatives

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Sidestream cigarette smoke solution was previously screened to identify the groups of chemicals in smoke that inhibit growth and angiogenesis in the chick chorioallantoic membrane (CAM). Pyrazine and several pyrazine derivatives were identified as a major chemical group in this screen. In the current study, purified pyrazine and six pyrazine derivatives identified in the screen were tested in dose response experiments to measure their effects on CAM growth, embryo growth, and angiogenesis. Chemicals or control medium were placed on CAMs in ovo on day 5 of development, and results were evaluated on day 6. Of the chemicals tested, pyrazine was the most potent and inhibited both CAM and embryo growth at picomolar doses. 2-Ethylpyrazine and 2,3-dimethylpyrazine were inhibitory at nanomolar doses. Inhibition of growth by pyrazine was correlated with inhibition of DNA synthesis. The pattern of blood vessel development in CAMs was disturbed by micromolar doses of pyrazine and 2,3-dimethylpyrazine. Migration of mesodermal blood vessels to the ectoderm of CAMs and their subsequent differentiation into the capillary plexus was impaired by nanomolar doses of pyrazine. In summary, these data show that pyrazine and some of its derivatives inhibit growth and certain processes important in angiogenesis at very low doses. Since pyrazine and some of its derivatives are considered safe food additives, further toxicological testing of pyrazine, in particular on developing tissues, should be done to fully evaluate its safety as a consumer product additive.

Cigarette smoke contains over 4,000 chemicals, some of which are well-characterized toxicants and carcinogens (EPA, 1992). Most of these compounds are present in both mainstream and sidestream smoke, although their relative amounts may vary significantly in these two forms of smoke (EPA, 1992). Mainstream smoke is the bolus of smoke inhaled by active smokers, while sidestream smoke burns off the end of cigarettes and is the main component of environmental tobacco smoke. Thus both active and passive smokers are exposed to numerous chemicals, most of which have not been well studied for their effects on developing tissue and organisms.

It is well known that smoking during pregnancy can cause a decrease in fetal weight (Stillman et al., 1986), and while this effect has been observed mainly in active smokers, it has also been reported in fetuses born to women exposed to environmental tobacco smoke during pregnancy (Martin and Bracken, 1986). We have previously shown that chemicals in both mainstream and sidestream cigarette smoke significantly impair growth of the chick chorioallantoic membrane (CAM) in a dose-dependent manner (Melkonian et al., 2002). The CAM is an extraembryonic membrane that begins forming between days 4 and 5 of development by fusion of the allantois and chorion (DeFouw et al., 1989; Hamilton, 1965). The CAM is of physiological importance to the chick, as it serves as the major respiratory organ for gaseous exchange until hatching, and it provides a “bladder” into which waste products can be delivered (Hamilton, 1965; Romanoff, 1967). Because the CAM increases 10–20-fold in size between days 5 and 6 of development, it provides a rapid and useful assay for evaluating the effects of chemicals on tissue growth (Melkonian et al., 2001, 2002). The CAM is also a widely used model for studying angiogenesis (Melkonian et al., 2001; Ribatti and Vacca, 1999).

In the CAM assay, both mainstream and sidestream smoke solutions caused very significant retardation of CAM growth between days 5 and 6 of development and also impaired various aspects of angiogenesis (Melkonian et al., 2002). The active chemicals partitioned mainly in the particulate phase of mainstream smoke and in the gas phase of sidestream smoke (Melkonian et al., 2002). To identify the chemicals responsible for retarding growth and angiogenesis in CAMs, we previously used a combination of solid phase extraction cartridges and gas chromatography-mass spectrometry to fractionate and identify the chemicals in sidestream gas phase smoke that inhibit CAM growth (Ji et al., 2002). Using this approach, 12 pyridine and 10 pyrazine derivatives were identified in the active fraction of sidestream gas phase smoke solutions. We previously tested the pyridines using the CAM assay and found that 2- and 3-ethyl pyridine inhibit CAM growth at picomolar doses (Ji et al., 2002).

The purpose of the present study was to test the pyrazine derivatives identified in the sidestream gas phase smoke solutions in the CAM assay to evaluate the effects of each on CAM growth and angiogenesis. In addition, the embryos of treated CAMs were weighed to establish if treatment with the test...
Material and Methods

Media and reagents. All buffers, including phosphate buffered saline (PBS), Earle’s Balanced Salt Solution (EBSS), and cadocylate were made using Barnstead/Thermolyne nanopure water (Fisher Scientific, Tusin, CA). EBSS was made fresh daily from a 10× stock solution. To a 1× salt solution, sodium bicarbonate and HEPES were added to make EBSS-HEPES (EBSS-H) (Talbot et al., 1998). The pH of EBSS-H was adjusted to 7.4 with NaOH and was used as the control solution to dilute test chemicals in all experiments. Glutaraldehyde (50%) was purchased from Electron Microscopy Supplies (Fort Washington, PA) and diluted to 3% in 0.1 M cacodylate buffer (pH 7.4) for use in fixation. Pyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2,5-di- methylpyrazine, 2,6-dimethylpyrazine, 2 methylpyrazine, and 2,3,5-trimethylpyrazine were purchased from Aldrich Chemical Company (Milwaukee, WI). Smoke solutions were made from research grade 2R1 cigarettes (University of Kentucky, Lexington, KY), as described in detail previously (Knoll and Talbot, 1998).

Solid phase extraction of smoke solutions. Bond-Elut solid phase extraction (SPE) cartridges (5cc with 500 gram capacity) (Phenomenex Torrance, CA) were used to fractionate smoke solutions and concentrate chemicals that inhibit CAM growth. Cartridges which were screened for their ability to bind inhibitory chemicals included a variety of non-polar, polar, and anion and cation exchange cartridges: NH2, 2OH, CN, CBA, SCX, SAX, C18, C8, C2, CH, SI, and PH. The protocol used to screen the cartridges for their ability to bind growth inhibitory toxianents in smoke solutions has been described in detail previously (Ji et al., 2002).

Gas chromatography-mass spectrometry (GC-MS). The C2 solid phase extraction cartridge retained most of the inhibitory activity in sidestream gas phase smoke solutions as determined in the CAM growth assay. To identify the components in aqueous smoke solutions that inhibit growth, mainstream and sidestream smoke solutions were analyzed with GC-MS after solid phase extraction on a C2 column. The equipment used was a Hewlett Packard 5890 GC interfaced to an HP-5971A MSD (quadrupole mass selective detector) with a Zebron ZB1701 cyanopropyl phenyl column 30 m × 0.32 mm and were made with a 1 μm phase thickness (Phenomenex, Torrance, CA.). The carrier gas was helium and the instrument was operated in the scanning mode (40–350 amu). The temperature program was an initial temperature of 45°C for one minute, with an increase of 10°C per minute to a final temperature of 280°C after ten min, with a total run time of 34.5 min. Two microliters of the eluate sample were injected directly into the GC using a Hamilton gas-tight syringe. Identification of compounds was made using the mass spectrometry data matched to mass spectral library entries. Compound identities were confirmed using purified standards and matching both mass spectra and retention times.

CAM assay and embryo wet weights. CAM growth was evaluated using a modification of the CAM assay that has been described in detail previously (Melkonian et al., 2001). 200 μl of either EBSS-H or different concentrations of pure chemicals were added to the surface of each CAM at 10 A.M. on the fifth day after fertilization. A sham control group, in which the window was opened and resealed, was also included in each experiment. In all cases, the following five concentrations of test reagents were used 5 × 10⁻⁷ M, 5 × 10⁻⁸ M, 5 × 10⁻⁹ M, 5 × 10⁻¹⁰ M, and 5 × 10⁻¹¹ M. Development was terminated at 10 A.M on the sixth day after fertilization by fixing the embryo and the CAM with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 hours at room temperature. The CAMs were later dissected from eggs, post-fixed in the same fixative for 24 hours at room temperature, then thoroughly rinsed in PBS. Embryos were removed from each egg and dissected free of any surrounding tissue before obtaining their wet weights. Each control and treatment group had at least five CAMs and five embryos.

Quantification and imaging of CAMs. To determine area, each CAM was placed in a Petri dish with PBS and examined with a Wild-MSA dissecting microscope (Max ERB instrument Co., Burbank, CA). The longest and shortest lengths were measured with a ruler to the precision of 0.5 mm, and CAM area was calculated using the formula:

\[ \text{Area} = (1/2 \times A) \times (1/2 \times B) \times \pi \]

Where A = longest length, B = longest width, \(\pi = 3.14\).

Means and standard deviations were then calculated for each control and treatment group. The treated group means were compared with control means to determine the effect of each chemical on CAM growth. The lowest observed adverse effect level (LOAEL), the no observed adverse effect level (NOAEL), and the maximum percentage of inhibition (efficacy) were determined for each pyrazine derivative.

Measurement of DNA synthesis. To determine if treatment of CAMs with pyrazine affected synthesis of DNA, day 5 CAMs were exposed to 5 × 10⁻⁷ M pyrazine for 21 hours, then 100 μl of H3-thymidine was added to each CAM. After 2 hours, CAMs were dissected from eggs, washed thoroughly several times in EBSS-H, and solubilized overnight in 8 M potassium hydroxide. Aliquots (20 μl) of the CAM lysate were added to 4 ml of scintillation cocktail, and counts were made 24 hours later using a Beckman scintillation counter.

Evaluation of blood vessel pattern formation in developing CAMs. Blood vessels in day 6 CAMs have a characteristic dendritic branching pattern (Melkonian et al., 2001). To evaluate blood vessel pattern formation, fixed CAMs were further dissected to remove edge tissue and mounted in tissue culture dishes under cover slips to flatten the CAMs. Video images were captured at a magnification of approximately 3× using a Hitachi KP-D50U camera (Hitachi Inc., Torrance, CA). To evaluate the effect of chemicals on pattern formation, blind comparisons of images of control and treated CAMs were made to a representative control image. The images were ranked from “0” to “2,” with “0” representing the branching pattern observed in controls and “2” representing the most severe disruption of blood vessel pattern. Images showing examples of disruption of pattern formation have been published previously (Melkonian et al., 2002).

CAMs that had been treated with pyrazine were embedded in plastic and processed for light microscopy as described previously (Melkonian et al., 2001). To evaluate capillary plexus formation, histological cross sections through CAMs were digitized with a Spot camera (Diagnostic Instruments, Sterling Heights, Michigan) using a 16× objective. For each parameter, one histological section from five different CAMs was examined at each dose group. To determine the percentage of ectoderm subtended by capillary plexus, capillary blood vessels that did not migrate to the basal lamina beneath the ectoderm was also counted for the same 600-μm region of each CAM.

Statistical analyses. Group means in each of the assays were compared by analysis of variance (ANOVA). When significance was found (p < 0.05), Dunnett’s post hoc test was used to identify significantly affected groups. Dunnett’s test compares treated group means to the EBSS-H control group. Data were checked to determine if they satisfied the assumptions of ANOVA (homogeneity of variances and normal distribution). If the assumptions were not satisfied, the Kruskal-Wallis non-parametric test was used followed by Dunn’s post hoc test. Analyses were done using GraphPad (Instat, San Diego, CA).
RESULTS

Pyrazines Identified in the Active Fractions of Sidestream Smoke Solutions

Seven pyrazine compounds that were identified by mass spectrometry in sidestream gas phase smoke solutions were available commercially in pure form and were tested in the CAM assay. These included pyrazine, 2-ethylpyrazine, 2,3-dimethylpyrazine, 2,5-dimethylpyrazine, 2,6-dimethylpyrazine, 2-methylpyrazine, and 2,3,5-trimethylpyrazine. The purity of the purchased pyrazines was confirmed using mass spectrometry before use in the dose response experiments. Four pyrazines were identified in sidestream gas phase smoke but could not be purchased commercially and therefore were not tested, and these included: 2,4-dimethylpyrazine, ethenyl pyrazine, 3-ethyl, 2,5-dimethyl pyrazine, and 5-ethyl, 2,3-dimethyl pyrazine. The results for the pyrazines that were used are reported in Figures 1–7 and are summarized in Table 1.

Pyrazine Inhibited Both CAM and Embryo Growth at Picomolar Doses

Compared to control CAMs that were treated with vehicle (EBSS-H) alone, pyrazine inhibited the growth of both the CAM and embryo in a dose-dependent manner (Figs. 1A,B). Significant inhibition of growth occurred at picomolar doses ($5 \times 10^{-11}$ M) in both assays. It is possible that significance was not detected in the $5 \times 10^{-13}$ M group, as it had only four CAMs. The maximum percentage of inhibition occurred at $5 \times 10^{-5}$ M and was 76% and 51.6% for the CAM and embryo, respectively.

A Single Ethyl Substitution to the Pyrazine Ring Slightly Decreased Its Potency

One pyrazine compound with a single ethyl substitution (2-ethylpyrazine) was identified in the active fraction of sidestream smoke. Like pyrazine alone, it was highly inhibitory in both the CAM and embryo growth assays (Figs. 2A,B). A dose dependent decrease in CAM and embryo growth was observed over the concentration range of $5 \times 10^{-11}$ to $5 \times 10^{-5}$ M. Significant inhibition of CAM and embryo growth was observed at nanomolar doses ($5 \times 10^{-9}$ M). The maximum percentage of inhibition was 51% and 26% for the CAM and embryo, respectively.

FIG. 1. Pyrazine inhibited both CAM (A) and embryo (B) growth at picomolar doses. (A) $5 \times 10^{-11}$ M to $5 \times 10^{-5}$ M pyrazine produced a significant dose dependent decrease in CAM growth as measured by CAM area in mm$^2$. (B) Embryo growth as measured by wet weight in grams was significantly inhibited compared to the EBSS treated control at the same doses. Each group had five CAMs, except for the $5 \times 10^{-13}$ M group, which had four. Values are plotted as means ± standard deviation. *p < 0.05, **p < 0.01.

FIG. 2. 2-Ethylpyrazine inhibited both CAM (A) and embryo (B) growth at nanomolar doses. (A) $5 \times 10^{-9}$ M to $5 \times 10^{-5}$ M 2-ethylpyrazine produced a significant dose dependent decrease in CAM growth as measured by CAM area in mm$^2$. (B) Embryo growth as measured by wet weight in grams was significantly inhibited compared to the EBSS treated control at the same doses. Each group had five CAMs. Values are plotted as means ± standard deviation. *p < 0.05, **p < 0.01.
A Single Methyl Substitution to the Pyrazine Ring Further Decreased Its Potency

2-Methylpyrazine was present in the active fraction of side-stream smoke. It significantly inhibited CAM growth at 5 × 10^{-5} M, and at this dose inhibition was 25% of the control (Fig. 3A). Embryo growth was not significantly affected at any dose tested (Fig. 3B).

Double Methyl Substitutions to Pyrazine Produced Mixed Effects on CAM and Embryo Growth

Three dimethylpyrazines identified in active fractions of smoke were tested in the CAM assay. 2,3-Dimethylpyrazine significantly inhibited CAM and embryo growth at doses as low as 5 × 10^{-7} M and 5 × 10^{-5} M, respectively (Figs. 4A,B). 2,5-Dimethylpyrazine was significantly inhibitory at 5 × 10^{-7} M in the CAM growth assay but did not significantly inhibit embryonic growth at any dose tested (Figs. 5A,B). 2,6-Dimethyl pyrazine did not inhibit growth of the CAM or embryo at any dose tested and was the only compound that stimulated growth in either assay (Figs. 6A,B). At all doses tested, embryo growth was slightly greater than in the EBSS-H treated control, and at 5 × 10^{-7} M 2,6-dimethyl pyrazine, the difference between the control and treated group was significant. The maximum percentage of inhibition in the CAM growth assay was 59% and 55% for 2,3-dimethylpyrazine and 2,5-dimethylpyrazine, respectively. The maximum percentage of inhibition of embryo growth was 21% for 2,5-dimethylpyrazine.

Triple Methyl Substitution Had a Small Inhibitory Effect on Embryo Growth

2,3,5-Trimethylpyrazine did not significantly affect CAM growth when compared to the control at any dose; however, inhibition in growth was observed at the highest concentration (5 × 10^{-5} M) (Fig. 7A). In the embryo assay, a small (19%) but significant decrease in growth was detected at 5 × 10^{-5} M (Fig. 7B).

Pyrazine Treatment Inhibits DNA Synthesis

Since pyrazine had the greatest potency and efficacy of the chemicals tested, it was studied further. To determine if pyrazine treatment inhibited CAM growth by inhibiting DNA synthesis, day 5 CAMs were treated with 5 × 10^{-7} M pyrazine, then 21 h later were labeled with H^{3}-thymidine as described in Materials and Methods. CAMs that were treated with pyrazine incorporated significantly less H^{3}-thymidine than the EBSS

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**FIG. 3.** 2-Methylpyrazine inhibited CAM (A) but not embryo (B) growth at micromolar doses. (A) 5 × 10^{-5} M 2-methylpyrazine produced a significant dose dependent decrease in CAM growth as measured by CAM area in mm². (B) Embryo growth as measured by wet weight in grams was not significantly inhibited at any of the doses tested. Each group had five CAMs. Values are plotted as means ± standard deviation. *p < 0.05, **p < 0.01.

**FIG. 4.** 2, 3-Dimethylpyrazine inhibited CAM (A) and embryo (B) growth at nanomolar and micromolar doses, respectively. (A) In the range of 5 × 10^{-7} M to 5 × 10^{-5} M, 2, 3-dimethylpyrazine produced a significant dose dependent decrease in CAM growth as measured by CAM area in mm². (B) Embryo growth as measured by wet weight in grams was significantly inhibited compared to the EBSS treated control at 5 × 10^{-5} M. Each group had five CAMs. Values are plotted as means ± standard deviation. *p < 0.05, **p < 0.01.
controls (Fig. 8), indicating that DNA synthesis was reduced by the treatment.

Effect of Pyrazine and Its Derivatives on Blood Vessel Pattern Formation

As blood vessels develop in the CAM, they form a characteristic dendritic branching pattern that can be disrupted by treatment with smoke solutions (Melkonian et al., 2001, 2002). Disruption of normal branching was evaluated in a blind test as described previously (Melkonian et al., 2002). Of the chemicals tested, only pyrazine and 2,3-dimethylpyrazine disrupted blood vessel pattern formation in CAMs, and disruption only occurred at the highest dose tested (5 \times 10^{-9} M).

Treatment of CAMs with Pyrazine Disrupts Formation of the Capillary Plexus

The capillary plexus normally begins forming during days 5 and 6 of chick development. By day 6, a discrete plexus underlies most of the ectoderm (Fig. 9A, arrows). This plexus originates mainly from migration of mesodermal blood vessels to the ectoderm, with some contribution from vascularization or formation of new vessels from angiogenic clusters just beneath the ectoderm (Melkonian et al., 2001). In histological sections of control CAMs treated with EBSS-H, the plexus was well formed, and most of the ectoderm had plexus vessels directly touching it (Fig. 9A). In CAMs treated with pyrazine, the plexus usually did not appear well developed and many mesodermal vessels (arrowheads) were still evident (Fig. 9B).

To determine if there existed a quantitative difference in the amount of plexus formed in treated versus control CAMs, the percentage of ectoderm with plexus immediately beneath it was computed for the pyrazine experiment (Fig. 10A). There was a dose-dependent decrease in the amount of plexus formed. Concentrations as low as 5 \times 10^{-9} M significantly inhibited formation of the plexus. To determine if this inhibition of plexus formation was related to failure of mesodermal blood vessels to migrate to the ectoderm, the number of mesodermal vessels was counted for each CAM (Fig. 10B). A dose-dependent inhibition of vessel migration by pyrazine was observed. Significant inhibition of migration occurred at 5 \times 10^{-9} M pyrazine. While the 5 \times 10^{-9} M treatment group likewise appeared to show inhibition, it was not significantly different than the control group.

DISCUSSION

Pyrazine and six pyrazine derivatives containing hydrocarbon substitutions were identified in fractions of sidestream
smoke solution. Pyrazine and these derivatives, when tested individually, inhibited growth of developing extra-embryonic membranes and embryos and disturbed some aspects of angiogenesis. Moreover, pyrazine and several of its derivatives were effective at pico and nanomolar doses in these assays. Pyrazine appeared to impair CAM and embryo growth by inhibiting cell proliferation, since DNA synthesis was significantly decreased by pyrazine. The LOAELs, NOAELs, and efficacy (maximum percentage of inhibition) for each compound tested in this study are compared in Table 1 for the CAM and embryo assays. The hierarchy of potency for the compounds tested based on their LOAELs in these assays is: pyrazine < 2-ethylpyrazine < 2,3-dimethylpyrazine < 2,5-dimethylpyrazine < 2-methylpyrazine < 2,3,5-trimethylpyrazine < 2,6-dimethylpyrazine. All tested pyrazines were inhibitory in these assays except for 2,6-dimethylpyrazine, which was not inhibitory at any dose, and in fact caused a small but significant stimulation of embryo growth at \(5 \times 10^{-9}\) M. Aspects of angiogenesis were likewise sensitive to pyrazine and its derivatives. Pyrazine and 2,3-dimethylpyrazine both impaired blood vessel pattern formation at \(5 \times 10^{-5}\) M. Pyrazine inhibited formation of the capillary plexus at doses as low as \(5 \times 10^{-9}\) M, indicating that plexus formation was more sensitive to pyrazine than vessel pattern formation.

The pyrazine ring inhibited both CAM and embryo growth more effectively than any of its derivatives. Substitution of an ethyl functional group on pyrazine decreased its potency 100-fold, while substitution of one or more methyl groups further decreased its potency by 1000- to 100,000-fold in the CAM/embryo growth assay. There was not a clear correlation with

**FIG. 7.** 2,3,5-Trimethylpyrazine inhibited embryo (B) but not CAM (A) growth at micromolar doses. (A) Although \(5 \times 10^{-5}\) M 2,3,5-trimethylpyrazine did produce a decrease in CAM area, it was not significantly different than the EBSS control. (B) Embryo growth as measured by wet weight in grams was significantly inhibited at \(5 \times 10^{-5}\) M. Each group had six CAMs. Values are plotted as means ± standard deviation. *p < 0.05, **p < 0.01.

**FIG. 8.** Pyrazine inhibits DNA synthesis. The counts per minute (CPM) of H3-thymidine incorporated into DNA are shown for pyrazine treated \((5 \times 10^{-7}\) M) and EBSS control CAMs. Each group had six CAMs. Values are plotted as the mean ± standard deviation. *p < 0.05

### TABLE 1

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<th>Chemical tested</th>
<th>NOAEL (M)</th>
<th>LOAEL (M)</th>
<th>Maximum % inhibition</th>
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</tr>
<tr>
<td>CAM</td>
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<td>(5 \times 10^{-10})</td>
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</tr>
<tr>
<td>CAM</td>
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<td>(5 \times 10^{-9})</td>
<td>51%</td>
</tr>
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<td>Embryo</td>
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<td>(5 \times 10^{-9})</td>
<td>26%</td>
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* Chemicals are listed in descending order based on the LOAEL in the CAM growth assay.

** 2,6-dimethylpyrazine did not inhibit CAM or embryo growth at any concentration; however it did slightly stimulate growth of the embryo at \(10^{-8}\) M.
the type of substitution and the level of inhibition as was seen previously for the pyridine derivatives in smoke (Ji et al., 2002), nor was there a correlation with lipophilicity (not shown). In a previous study in which pyridine derivatives were screened in the CAM growth assay, pyridine itself was not strongly inhibitory. However, addition of a single methyl group increased its potency by 10 thousand-fold and addition of a single ethyl group increased its potency by 10 million-fold (Ji et al., 2002). In contrast to the pyridines, the data with the pyrazines indicate that the effect of pyrazines on growth cannot necessarily be predicted from their structure and that each chemical needs to be evaluated individually to determine its effect on tissue growth. It is interesting that pyrazine differs from pyridine only by the presence of a second nitrogen atom in the fourth position of the ring. This simple modification to the ring resulted in a 1 million-fold increase in the potency of pyrazine (LOAEL = $5 \times 10^{-11}$ M) versus pyridine (LOAEL = $5 \times 10^{-5}$ M) in the CAM growth assay.

The data for 2,5-dimethylpyrazine are interesting in that they show a very significant decrease (over 50% inhibition) in CAM growth at $5 \times 10^{-7}$ and $5 \times 10^{-5}$ M, while the same doses produced no significant effect on embryo growth. These data indicate that a smaller CAM does not necessarily produce a smaller embryo. 2,5-Dimethylpyrazine was the only chemical that specifically inhibited CAM growth without affecting embryo growth, which further shows that the effects of these chemicals are complex and may depend on the type of tissue being studied. These data also suggest that the chemicals that inhibited both CAM and embryo growth probably inhibited embryo growth by acting directly on the embryos, as decreased CAM size did not necessarily correlate with decreased embryo size.

Angiogenesis is complex, involving numerous cell processes (Folkman and Shing, 1992; Risau, 1997). In this study, pyrazine and 2,3-dimethylpyrazine altered normal branching of blood vessels in the developing CAMs. However, the other pyrazines were without significant effect in this assay at the
highest dose tested (5 × 10⁻⁵ M). Since whole smoke produces a strong effect on vessel branching (Melkonian et al., 2000, 2002), the data obtained with pyrazine and 2,3-dimethylpyrazine suggest that there may be other chemicals in smoke that are more active in this assay. Pyrazine also inhibited the migration of mesodermal blood vessels to the ectoderm and the subsequent formation of the capillary plexus at nanomolar doses. This observation shows that pyrazine can retard at least one process important in angiogenesis at low doses. Failure of the capillary plexus to form properly in pyrazine-treated CAMs could compromise oxygen and CO₂ exchange and may, like inhibition of DNA synthesis, also be a factor contributing to retarded growth of CAMs and embryos.

The biological effects of pyrazine and its derivatives are not well understood. In fact, pyrazine plus several of its derivatives appear on a list of chemicals published by the Flavor and Extract Manufacturers Association (FEMA) that are generally recognized as safe (GRAS) and are often added to consumer products such as food, tobacco, cosmetics, and fragrances (Adams et al., 2002; Smith et al., 2001). Pyrazine, which was added to this list in December 2001, may be the most significant with respect to our data. Of the compounds we tested, pyrazine was the most potent at inhibiting CAM and embryo growth, having a LOAEL of 5 × 10⁻¹¹ M in both the CAM and embryo assays. In the CAM growth assay, the LOAEL and estimated ED₉₀ (5 × 10⁻⁷ M) doses are equivalent to 4 ppb and 0.4 ppm, respectively. The ED₉₀ dose in ppm is within the range of concentrations for the average usual (0.3–1 ppm) and average maximum (1.5–5 ppm) concentration of pyrazine added to various types of food (Smith et al., 2001). The LOAEL dose (4 ppb) is, of course, well below the concentrations of pyrazine that are added to food products.

In our study of developing tissues, pyrazine had a profound effect on growth in both the CAM and embryo assay. Most toxicological studies on pyrazine and its derivatives have involved adults, and effective doses may be different for adult tissue and developing tissue.

However, there is evidence from both in vitro and in vivo models that supports the idea that pyrazine and its derivatives can inhibit growth in adults. In vitro studies with cultured cells have shown that growth can be inhibited by pyrazine derivatives (Shang et al., 1998; Zurbonson et al., 1999). Moreover, in some prior studies done in vivo using adult rodent models, the weight of the animals or of specific organs decreased following oral exposure to a pyrazine derivative (Adams et al., 2002; Posternak et al., 1969, 1975). In some instances, this was attributed to test animals eating less and therefore gaining less weight (Posternak et al., 1969, 1975), although intake of food was not actually quantified in all studies. In some in vivo studies, body weight or specific organ weight was compromised by exposure to pyrazines that were administered as vapors (Katz et al., 1999) or by subcutaneous injection (Yamada et al., 1996). Reproductive organs can be targets of growth inhibition, as administration of 2,5-dimethylpyrazine to female rats caused a significant reduction in uterine, but not ovarian, weight, perhaps by affecting uptake of estradiol by the uterus (Yamada et al., 1992). Reproductive processes appear also to be sensitive to pyrazine derivatives, as 2,5-dimethylpyrazine decreased the overall success of reproduction for rodents that were housed together, although high doses (>70 mg/kg body weight/day) were required to observe this effect (Novotny et al., 1986). Moreover, first vaginal opening in rats, which is an indicator of the onset of puberty, was also inhibited by tetramethyl and 2,5-dimethylpyrazine (Yamada et al., 1989). However, in one study on reproduction, administration of tetramethylpyrazine produced no significant effect on any reproductive parameter that was monitored (Vollmuth et al., 1990). In our study, the LOAELs of the tested pyrazines were quite different, and the derivative with the most substituted groups (2,3,5-trimethylpyrazine) was not as effective at inhibiting CAM growth as pyrazine and 2-ethylpyrazine.

The implications of our data for human reproduction are at present not known, but well-established information on smoking and growth during reproduction indicate that further work should be done to address this topic. Establishing and maintaining pregnancies in mammals requires extensive growth in the embryo/fetus, corpus luteum, and the placenta (Findlay, 1986), and this growth can be compromised by exposure to cigarette smoke (Stillman et al., 1986). Both active (Stillman et al., 1986) and passive (Martin and Bracken, 1986) smokers have fetuses with lower than normal birth weights. This reduction in birth weight is thought to be due to fetal hypoxia caused by nicotine-induced vasoconstriction and decreased placental blood flow and by increased carboxyhemoglobin leading to decreased fetal oxygenation (Tourmaa, 1995). Given the complexity of cigarette smoke, it is likely that multiple mechanisms affect prenatal growth in smoke-exposed fetuses. We have found that both ethyl and methyl substituted pyridines (Ji et al., 2002), pyrazine, and 2-ethylpyrazine are very effective at retarding growth in the CAM and embryo assay at very low doses and that, for pyrazine, this inhibition correlates with inhibition of DNA synthesis. Since pyrazine and several of its derivatives are present in cigarette smoke and are added to consumer products, further work on the toxicity of these pyrazines, especially in developing organisms, should be undertaken to obtain a more complete understanding of their effect on human health and reproduction.

REFERENCES


