Identification of Hypoxanthine and Inosine in Brain Dialyzable Fraction as Stimulators for Growth of Porcine Aortic Endothelial Cells in Response to Fibroblast Growth Factor in Either Dialyzed Serum Media or Low Serum Media

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Abstract—The rate of proliferation of porcine aortic endothelial cells (PAEC) in response to fibroblast growth factor (FGF) was largely retarded when incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with either 1% fetal bovine serum (FBS) or 10% dialyzed FBS in place of 10% FBS. Proliferation of endothelial cells in low serum media in response to FGF was enhanced to the level of media containing FGF plus 10% FBS by the addition of the dialyzable fraction from bovine brain homogenates. From the bovine brain dialyzable fraction, two active components were purified and identified as hypoxanthine and inosine. Either hypoxanthine or inosine, at a dose of 5 µM in DMEM with 1% FBS, maximally increased the incorporation of [3H]thymidine into DNA of PAEC in low serum media in the presence of FGF. However, no additive effect was observed when hypoxanthine and inosine were added simultaneously. The present data indicate that the proliferative action of FGF on PAEC can be potentiated by hypoxanthine and inosine.

Fibroblast growth factors (FGF) are constantly present in normal central nervous tissues such as the brain (1) and pituitary (2); however, neovascularization and proliferation of endothelial cells in these tissues are very low (3). Therefore, it has been suspected that the onset of FGF action in central nervous tissues is negatively controlled by some components. Folkman et al. have indicated that basic FGF (FGFb) activity is masked by its binding to heparan sulfate localized in the basement membrane of the bovine cornea (4). Heparin is known to inhibit FGFb activity during endothelial cell proliferation in vitro (5). In contrast to these macromolecular inhibitors, low molecular weight substances, such as corticoids (6) and endothelial cell stimulating angiogenesis factor (ESAF) (7), have been reported to stimulate endothelial cell proliferation in response to FGF. Very recently, we demonstrated that FGF-action on porcine aortic endothelial cell (PAEC) growth was largely retarded when the cells were incubated in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1% fetal bovine serum (FBS) or 10% dialyzed FBS (8). Using this model, we have attempted to identify stimulatory or inhibitory effectors of PAEC growth, particularly in response to FGF, from the bovine brain dialyzable fraction.

Materials and Methods

Cell culture
Porcine aortic endothelial cells (PAEC) were isolated and grown as described (9). Briefly, the PAEC were grown in DMEM containing 10% FBS and subcultured into a 25-cm² culture flask with a 0.25% trypsin solution containing 0.02% ethylenediaminetetraacetic acid (EDTA) when the cells reached con-
fluence. Generally, cells from passages 8 to 12 were used for testing purposes. In the cells cultured in 1% FBS or 10% dialyzed FBS, the percentage of non-viable cells was less than 5%.

Thymidine incorporation assay

Cells (1 × 10^4/cm²) were seeded in 48-well (Costar) tissue culture plates and then incubated for 32 hr in 500 ml of media containing 10% FBS to allow for attachment. The medium was then replaced with fresh DMEM containing 1% FBS with or without bovine brain dialyzable fraction or its extract, and/or an aliquot of acidic FGF (FGFa) plus heparin or FGFb. After incubating for 16 hr, all the wells were pulsed for 8 hr with 0.1 μCi of [3H]thymidine (10 μM) per well. The cells were then washed with cold phosphate-buffered saline and solubilized with 0.2 ml of 0.25 M NaOH. [3H]Thymidine-incorporated DNA was precipitated with 10% trichloroacetic acid. The resulting precipitates were collected on glass filters (GF/F, Whatman), and the filters were washed with 10% trichloroacetic acid and ethanol. Radioactivity on the filter was counted by a liquid scintillation counter using 5 ml of toluene containing 0.5% 2,5-diphenyloxazole.

Rate of proliferation assay

For measuring the number of viable cells, PAEC (4 × 10^4) were grown in 6-well (9.6 cm²) tissue culture plates (Becton Dickinson Lab., U.S.A.). After incubating for various times, the number of detached cells was counted with a hemocytometer in a trypsin (0.25%) - EDTA (0.02%) solution following staining with 0.05% nigrosine solution in saline. Replicate wells in the same dish were generally examined for any of the given test conditions.

Purification of hypoxanthine and inosine from dialyzable fraction of bovine homogenates

Preparation of dialyzable fraction of bovine brain homogenates: Fresh bovine brains (470 g) were homogenized in 470 ml of cold H₂O with a Waring blender at 4°C. Homogenates were dialyzed against 15 volumes (v/v) of cold H₂O for 18 hr. Dialyzable fractions obtained were concentrated by a freeze-dry method, and aliquots were adjusted by dissolving in distilled water so that 1 μl of the dialyzable fraction was derived from 1 mg of brain tissue (referred to as the bovine brain dialyzable fraction).

Isolation of active compounds from dialyzable fraction: The bovine brain dialyzable fraction was adjusted to pH 12 with 5 M NaOH and stirred with Dowex 1×8 (OH⁻ form) resin (56 ml in volume). After washing with 10 mM NaOH (100 ml), the resin was adjusted to pH 2.0 with aliquots of 1 M HCl, and packed in a column (3×8 cm). Then the column was rinsed with 10 mM HCl. The growth-promoting activity eluted in fractions was assayed by [3H]thymidine incorporation into DNA. The active fractions were combined and mixed with 5 g of silica gel (Merck, Kieselgel 60, 70–230 mesh). The gel suspension was evaporated and suspended in acetone. The acetone suspension was layered on top of fresh silica gel (26.6 ml in volume) in a column (2.2×10 cm). The activity for [3H]-DNA synthesis was eluted from the gel with 200 ml of a mixture of acetone : water (9:1, v/v), combined, and evaporated. The yield of the active compound was approximately 126 mg, which was readorsed on to 0.50 g of silica gel and rechromatographed (1.3×10 cm). Two peaks of [3H]DNA synthesis activity were eluted with a mixture of chloroform : methanol : water (4:2:1, v/v, a bottom phase). Pure active compound, 17.2 mg, was recovered from several fractions in the first peak, which was crystallized from hot water. Since the active fractions of the second peak (20.5 mg) were highly contaminated with the first peak component, they were combined and mixed with 0.2 g silica gel. After evaporating, the gel powder was layered on top of a fresh silica gel column (1×10.5 cm). The [3H]DNA synthetic activity was eluted from the gel with a mixture of chloroform : methanol : water (10:5:1, v/v). The yield of pure active component from the second peak was 4.09 mg, which was crystallized from hot water. The purity of each compound was checked by thin layer chromatography (TLC) on Kieselgel 60 F₂₅₄ plates (0.25 mm thickness) using a solvent system of chloroform : methanol : water (4:2:1, v/v, a bottom phase), and by high-performance liquid chromatography (HPLC) on an SC-02 column (JASCO, Japan) using a methanol
gradient of 0 to 40% in 0.02 M potassium phosphate buffer (pH 5.6). The active compounds on TLC plates were routinely visualized using three methods: 1) fluorescence quenching, 2) iodine adsorption, and 3) reaction with ninhydrin. Each spot visualized by these methods on plates was scrapped off and washed with aliquots of H2O to extract the components.

**Identification of chemical nature of active components:** Identification of pure active fractions was conducted by the following methods: 1H and 13C-nuclear magnetic resonance (NMR) spectra were recorded on a JNM-FX200 spectrometer (JEOL, Japan). NMR spectra were taken as 1-2% w/v solutions in dimethylsulfoxide-d6 (99.8%) at 26°C with tetramethylsilane as an internal reference. Infrared spectra were recorded with a Shimadzu IR-400 spectrophotometer. Electron impact and field desorption mass spectra were taken on a JMS-01 S6-2 spectrometer (JEOL, Japan).

**Statistical analysis**

Student's t-test was used to determine the significance of the difference between groups (10).

**Chemicals and reagents**

The chemicals and reagents used were obtained from the following sources: Dulbecco’s modified Eagle’s medium “Nissui” from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); fetal bovine serum from Flow Lab. (North Pyde, Australia); acidic fibroblast growth factor from bovine brain and basic fibroblast growth factor from bovine pituitaries from Toyobo Co. (Tokyo, Japan); [methyl-3H]-thymidine from Amersham Japan (Tokyo, Japan); Dowex 1 x 8 (200-400 mesh) from Dow Co. (Midland, MI, U.S.A); thin layer chromatography plates silica gel 60 F254, 0.25 mm thickness, and silica gel 60 for column chromatography (70-230 mesh) from Merck (Darmstadt, Germany); heparin and other reagents of the highest available quality were obtained from commercial sources.

**Results**

Effect of bovine brain dialyzable fraction on the rate of proliferation of PAEC: In media with 1% FBS or 10% dialyzed FBS, the number of growing PAEC decreased markedly as compared with that in the regular media containing 10% FBS. This cell growth retardation was found to be mostly overcome by the addition of 1% of the bovine brain dialyzable fraction into each media (Fig. 1). The brain dialyzable fraction, however, did not stimulate the proliferation of PAEC in media containing 10% FBS.

**Effect of bovine brain dialyzable fraction on the [3H]thymidine incorporation into DNA of PAEC in response to FGF:** The incorporation of [3H]thymidine into DNA of PAEC in low serum media was dose-dependently enhanced by increasing the amount of the bovine brain dialyzable fraction (Fig. 2). The stimulation reached a maximal level at 1% of the bovine brain dialyzable fraction.

Both FGFb and FGFa plus heparin increased the incorporation of [3H]thymidine into DNA of PAEC in media with 10% FBS, but not in media with 1% FBS. The addition of 1% bovine brain dialyzable fraction into media with 1% FBS markedly stimulated [3H]-thymidine incorporation into DNA of PAEC in...
Fig. 2. Dose-dependent effect of the bovine brain dialyzable fraction on the [3H]thymidine incorporation into DNA of PAEC. PAEC were incubated for 16 hr in media containing 1% FBS in the presence of various volumes of the bovine brain dialyzable fraction. All wells were pulsed for 8 hr with [3H]-thymidine, and then 3H-labeled DNA was extracted and counted. Doses of the brain dialyzable fraction are indicated as values of "% (v/v) in media". Each value is the mean of two samples.

Fig. 3. Effect of the bovine brain dialyzable fraction on [3H]thymidine incorporation into DNA of PAEC in response to FGFb or FGFa plus heparin. PAEC were incubated in media containing 1% FBS with or without 1% (v/v) of the bovine brain dialyzable fraction in the presence (hatched column) or absence (open column) of FGFa (10 ng/ml) with heparin (10 μg/ml) (A) or FGFb (10 ng/ml) (B). The incorporation of [3H]thymidine is indicated as a % of control. The control values (as 100%) were: 449±16 dpm/well (A) and 273±0.2 dpm/well (B). Each value is the mean±S.E. of three samples. Statistical significance, *P<0.001; N.S., not significant.

Fig. 4. Separation of two distinctive active components by second silica gel column chromatography. (A) A portion (10 μl) of each fraction (11–37) from the second silica gel column chromatography (eluant: chloroform/methanol/water, 4:2:1, v/v, the bottom phase) was used for silica gel thin layer chromatography (developing solvent: chloroform/methanol/water, 10:5:1, v/v). Lanes a, b and c contained hypoxanthine (4 μg), inosine (6 μg) and the pooled fractions from the first silica gel column chromatography (eluant: acetone/water, 9:1), respectively. Each spot was visualized using ultraviolet light (254 nm) and photographed. (B) Each part of lane c (in Fig. 4A) as indicated by horizontal bars was extracted with water for the measurement of the activity of [3H]thymidine incorporation into DNA of PAEC.
the presence of FGFα plus heparin (Fig. 3A) or FGFβ (Fig. 3B).

**Purification of hypoxanthine and inosine as active components from bovine brain dialyzable fraction:** The bovine dialyzable fraction was subjected to several steps of purification as described in Materials and Methods. Two active components (compounds 1 and 2) for stimulating the [3H]DNA synthesis of PAEC were fractionated by means of silica gel chromatography. Compounds 1 and 2 were identified to be hypoxanthine and inosine, respectively, from the results of their relative mobilities on TLC plates as compared with those of reference compounds (Fig. 4).

Tables 1, 2 and 3 summarize the physicochemical properties of purified active compounds. All analytical data for compounds 1 and 2 were assigned the structures of hypoxanthine and inosine, respectively; and they were completely consistent with data of the hypoxanthine and inosine standards.

**Effects of hypoxanthine and inosine on the [3H]DNA synthesis of PAEC:** Hypoxanthine and inosine dose-dependently stimulated the [3H]thymidine incorporation into DNA of PAEC in 1% FBS media with or without FGFα plus heparin (Fig. 5). Maximal growth of FGF-stimulated PAEC was obtained when 5 μM of hypoxanthine or inosine was supplemented into the media. However, no additive effect was observed on [3H]thymidine incorporation into DNA of PAEC in 1% FBS media with or without FGF when 50 μM of

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**Table 1. ¹H NMR chemical shifts of compounds 1 and 2**

| Proton | Compound 1 | Hypoxanthine | Compound 2 | Inosine |
|--------|------------|--------------|------------|---------|
| 2-H    | 8.13, s    | 8.13, s      | 8.34, s    | 8.38, s |
| 6-OH   | 11.5–13.5  | 11.5–13.5    | 11.3–12.5  | 12.43, br, s |
| 8-H    | 7.98, s    | 7.98, s      | 8.07, s    | 8.12, s |
| 1'-H   | 5.87, d (5.5)* | 5.91, d (5.9) |
| 2'-H   | 4.48, dd (5.1, 5.5) | 4.53, q (5.5) |
| 2'-OH  | 5.51, br, s | 5.53, d (5.8) |
| 3'-H   | 4.13, dd (3.7, 4.8) | 4.17, dt (4.0, 4.4) |
| 3'-OH  | 5.23, br, s | 5.24, d (4.8) |
| 4'-H   | 3.94, q (3.7) | 3.98, q (3.7) |
| 5'-Ha  | 3.65, dd (3.7, 12.1) | 3.70, ddd (4.4, 4.8, 12.1) |
| 5'-Hb  | 3.56, dd (2.9, 12.8) | 3.58, ddd (4.2, 5.7, 12.1) |
| 5'-OH  | 5.09, br, s | 5.11, t (5.5) |

*: The coupling constants (Hz) are in parentheses.

**Table 2. ¹³C NMR chemical shifts of compounds 1 and 2**

| Carbon | Compound 1 | Hypoxanthine | Compound 2 | Inosine |
|--------|------------|--------------|------------|---------|
| 2-C    | 144.5      | 144.5 (144.6)* | 145.8      | 145.8 (146.2) |
| 4-C    | 153.4      | 153.1 (153.2) | 148.1      | 148.1 (148.5) |
| 5-C    | 118.9      | 119.2 (119.2) | 124.3      | 124.4 (124.6) |
| 6-C    | 155.3      | 155.2 (155.4) | 156.4      | 156.4 (156.9) |
| 8-C    | 140.1      | 140.1 (140.2) | 138.6      | 138.6 (139.1) |
| 1'-C   | 87.4       | 87.4 (87.8)  | 87.4       | 87.4 (87.8)  |
| 2'-C   | 74.0       | 74.0 (74.4)  | 74.0       | 74.0 (74.4)  |
| 3'-C   | 70.2       | 70.2 (70.6)  | 70.2       | 70.2 (70.6)  |
| 4'-C   | 85.5       | 85.5 (88.9)  | 85.5       | 85.5 (88.9)  |
| 5'-C   | 61.2       | 61.2 (61.6)  | 61.2       | 61.2 (61.6)  |

*: The data for hypoxanthine and inosine, in parentheses, are from Chen et al. (28, 29).
Fig. 5. Effect of hypoxanthine or inosine on $^{[3}\text{H}]$thymidine incorporation into DNA of PAEC in response to FGFa plus heparin. PAEC were incubated for 16 hr in media containing 1% FBS with (closed symbols) or without (open symbols) FGFa (10 ng/ml) plus heparin (10 μg/ml), and with various doses of hypoxanthine (□) or inosine (△). To all wells were added $^{[3}\text{H}]$thymidine and incubated for a further 8 hr; then $^{[3}\text{H}]$DNA was extracted and counted by the procedures described in Materials and Methods. Each value is the mean±S.E. of three samples. Statistical significance, *P<0.001.

hypoxanthine and inosine were added simultaneously (Fig. 6).

Discussion

The present report shows that PAEC do not proliferate well in response to FGF in either dialyzed serum media or low serum media, and that proliferation of PAEC in low serum media in response to FGF can be enhanced to a level of media containing 10% FBS by addition of the brain dialyzable fraction. Active components of the dialyzable fraction were found to be hypoxanthine or inosine.

Although hypoxanthine and inosine both were demonstrated to be active in the stimulation of $^{[3}\text{H}]$DNA synthesis in PAEC in low serum media, their action mechanism may be
not so different from each other, because the maximum stimulation by hypoxanthine was not further enhanced by addition of an excess amount of inosine, and vice versa (Fig. 6). Inosine is hydrolyzed to yield its purine base hypoxanthine and D-ribose, whereas hypoxanthine is salvaged in conjunction with 5-phosphoribosyl-1-pyrophosphate (PRPP) to yield inosic acid by hypoxanthine-guanine phosphoribosyltransferase. Therefore, inosine may be hydrolyzed to hypoxanthine prior to being salvaged through this enzyme action, because free purines formed on degradation of purine nucleotides are in large part salvaged and used over again to remake nucleotides. Since FGFb was reported to stimulate the formation of PRPP in fibroblasts (11), it might be available for FGF-stimulated endothelial cells to reassemble purine nucleotides via the salvage pathway in PAEC in low serum media. However, there is less information about the metabolism of hypoxanthine, inosine or PRPP in PAEC in low serum media with or without FGF.

The bovine brain dialyzable fraction, hypoxanthine or inosine, mildly stimulated the \[^3H\] DNA synthesis of PAEC in low serum media in the absence of FGF (Figs. 3 and 5). This basal stimulation may be caused by the residual FGF in 1% FBS, because bovine serum is reported to contain approximately 200 ng/ml of immunoreactive FGF (12). On the other hand, the bovine brain dialyzable fraction did not induce more growth-promoting activity in PAEC cultured in 10% FBS in place of 1% FBS or 10% dialyzed FBS. If we assume hypoxanthine is an essential component in both basal and FGF-stimulated growth of PAEC, this result indicates that 10% FBS might contain a sufficient quantity of hypoxanthine. In fact, a high concentration of hypoxanthine, about 75 \( \mu \text{M} \), was reported in FBS (13), in contrast to a much lower level (1-2.5 \( \mu \text{M} \)) of hypoxanthine in bovine serum (13) and human plasma (14). Subsequently, the regular medium with 10% FBS contained more than 5 \( \mu \text{M} \) of hypoxanthine, and this concentration corresponded with the dose that produced maximal stimulation of \[^3H\] thymidine incorporation into DNA of PAEC (Fig. 5).

Furthermore, it is noteworthy that both hypoxanthine levels are high in tissues in which angiogenesis occurs such as ovarian follicle (15), placenta (16), wound sites (17), and tumors (18) as well as ischemic brain (19). These results including our present observation suggest that the increases in hypoxanthine and inosine levels have something to do with FGF-stimulated growth of endothelial cells (or angiogenesis).

Of particular importance is the work of Weiss and her collaborators. They showed that endothelial cell stimulating angiogenesis factor (ESAF), molecular mass of \(<600\) (20), potentiates the activity of FGF (7). Although the chemical nature of ESAF has not been determined, ESAF, in contrast to hypoxanthine, stimulated capillary endothelial cell growth, but not aortic endothelial cell growth (21). Furthermore, ESAF activated mammalian procollagenase (22), while hypoxanthine did not have such activity (Y. Hayashi et al., unpublished data). Therefore, we suspect that ESAF differs from the characteristics of hypoxanthine with regard to its biological activity.

Hypoxanthine has been reported to have various biological activities such as stimulation of DNA synthesis in serum-starved L-cells (23) and neuroblasts (24), stimulation of differentiation of erythroleukemia cells (25, 26), and maintenance of murine oocyte meiotic arrest (15), and enhancement of cellular immunity (27). Besides these actions, we present here that hypoxanthine and inosine have an effect on FGF-dependent proliferation of PAEC grown in a serum-limited culture system.

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