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FIBROBLAST GROWTH FACTOR-DEPENDENT METABOLISM OF HYPOXANTHINE VIA THE SALVAGE PATHWAY FOR PURINE SYNTHESIS IN PORCINE AORTIC ENDOTHELIAL CELLS

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Abstract—In this study we examined the metabolism of hypoxanthine in fibroblast growth factor (FGF)-stimulated porcine aortic endothelial cells (PAEC). Our previous report indicated that hypoxanthine in fetal bovine serum (FBS) was an essential component for both basal and FGF-dependent growth of PAEC (Hayashi *et al.*, *Exp Cell Res* **185**: 217–228, 1989). Besides hypoxanthine, the addition of various purine bases and purine nucleosides, but not xanthine, xanthosine or any pyrimidine metabolites, restored the limited growth of PAEC cultured in medium containing 10% dialyzed FBS in the presence or absence of FGF. The metabolism of [^{14}C]hypoxanthine was compared in PAEC treated with and without FGF. Treatment of PAEC with FGF for 24 hr enhanced the radioactivity incorporation from [^{14}C]hypoxanthine into both the acid-soluble and -insoluble fractions approximately 2-fold. Upon chromatographic analyses of hypoxanthine metabolites in the acid-soluble nucleotide fraction, it was found that in control PAEC hypoxanthine was largely metabolized to IMP, adenine nucleotides and uric acid, whereas in FGF-treated cells it was converted to ATP, ADP, GTP, xanthine and uric acid. The radioactivity of IMP was lowered in FGF-stimulated cells. The addition of FGF to PAEC increased phosphoribosyl pyrophosphate (PRPP) synthetase activity by approximately 8-fold and the PRPP content by approximately 2-fold, but it did not increase hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity or hypoxanthine transport. On the other hand, methotrexate, an inhibitor of *de novo* synthesis of purine, did not affect the growth of PAEC. Analyses of the rate of [^{14}C]formate incorporation into total purine compounds showed that PAEC had a low capacity to synthesize purines *de novo*, which was not stimulated by FGF. These data indicate that FGF stimulates the synthesis of PRPP necessary for the salvage synthesis of purine nucleotides in conjunction with purine bases, e.g. hypoxanthine.

Irregular purine nucleotide catabolism and extracellular release of purine metabolites occur in tissues during hypoxia [1, 2], ischemia and reperfusion [3]. On these occasions, the endothelium is thought to be one of the candidates that metabolize the released purine metabolites to purine nucleotides or nucleic acids [4]. Adenosine and hypoxia were demonstrated to stimulate the proliferation of bovine aortic endothelial cells *in vitro* [1]. In addition, we showed that hypoxanthine [5–7] and inosine [6], components extracted from the dialyzable fraction of fetal bovine serum (FBS)† [5, 7] and ischemia bovine brain tissue [6], enhance the proliferation activity in porcine aortic endothelial cells (PAEC). For basal and fibroblast growth factor (FGF)-dependent growth of PAEC, hypoxanthine was an essential factor when the PAEC were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed FBS. Furthermore, FBS contains a 100 times higher level of hypoxanthine than calf or newborn bovine serum, which means that FBS is much better as a supplement in the culture medium for PAEC growth in response to FGF [7]. These data reflect the special purine metabolism in the growth of PAEC in response to FGF.

Therefore, the aim of the present study was to characterize the coordinative effects of FGF and hypoxanthine on the growth of PAEC. Here we demonstrate that FGF triggers 5-phosphoribosyl 1-pyrophosphate (PRPP) synthesis, resulting in the increased synthesis of purine nucleotides and nucleic acids in association with hypoxanthine via a salvage pathway.

MATERIALS AND METHODS

Chemicals and reagents

The chemicals and reagents used were obtained from the following sources: DMEM from the Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); fetal bovine serum from Flow Lab. (North Pyde, Australia); acidic FGF (FGFa) from bovine brain and basic FGF (FGFb) from bovine pituitary from the Toyobo Co. (Tokyo, Japan); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and PRPP from Sigma (St. Louis, MO,

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† Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; FGFa, acidic fibroblast growth factor; FGFb, basic fibroblast growth factor; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PAEC, porcine aortic endothelial cells; and PRPP, 5-phosphoribosyl 1-pyrophosphate.

U.S.A.); [8-^{14}C]hypoxanthine, [^{14}C]formic acid and PROTOSOL® from NEN Research Products (Boston, MA, U.S.A.); POLYGRAM™ (thin-layer chromatography plates with 0.1 mm cellulose MN300 polyethyleneimine) from Macherey-Nagel (Postfach, Germany); and heparin, methotrexate and other reagents of the highest available quality from commercial sources.

Cell culture and proliferation assay

Cell culture. PAEC were isolated from a fresh porcine aorta and grown as described in our previous paper [5]. Briefly, the PAEC were grown in DMEM containing 10% FBS, and then subcultured in a 25-cm^2 culture flask with a 0.25% trypsin solution containing 0.02% EDTA when the cell reached confluence. Generally, cells from passages 8 to 12 were used for testing purposes. Dialyzed FBS was prepared by dialysis against 30 vol. of Hanks' solution at 4°, with two changes of the solution after 16 and 32 hr, for 48 hr, and then sterilized by filtration through a 0.22 μm Millipore filter. Fibroblasts were isolated from rat dorsal skin essentially according to the method of Hunter [8].

Cell proliferation assay. Cells (0.5×10^4 cells/cm^2) in 24-well (Falcon) tissue culture plates were incubated with 0.5 mL of medium containing 10% FBS or 10% dialyzed FBS plus the additions indicated. After 4 days of incubation at 37° in a CO_2-incubator, the cells were counted by a slightly modified version of the colorimetric MTT method [5], which was originally described by Mosmann [9].

Measurement of hypoxanthine metabolism in PAEC

[^{14}C]*Hypoxanthine metabolism.* PAEC (1×10^4 cells/cm^2) were seeded into 24-well tissue culture plates. After a 24-hr incubation, the medium was replaced by DMEM supplemented with 10% dialyzed FBS, and the cells were preincubated for 12 hr. Then either FGFa plus heparin or FGFb was added and the cells were further incubated for 1–24 hr (in a routine assay, 12 hr). Next the cells were labeled with [^{14}C]hypoxanthine (0.1 μCi/6 μM, final concentration, in 0.4 mL DMEM) at 37° for 1–24 hr (in a routine assay, 5 hr). Then the cells were washed with ice-cold phosphate-buffered saline, and the reactions stopped with 0.5 mL of ice-cold 0.6 M perchloric acid. The cells were scraped off with a rubber policeman. The acid-insoluble fraction was separated by centrifugation at 8000 *g* for 10 min, washed, solubilized with PROTOSOL®, and then counted with a liquid scintillation counter. The supernatant fluid was subsequently neutralized with 0.3 mL of 1 M $KHCO_3$ and then centrifuged, and the resulting clear supernatant (acid-soluble fraction) was counted with a liquid scintillation counter.

Analysis of radioactivity with HPLC and TLC. PAEC (1×10^4 cells/cm^2) were seeded into 6-well tissue culture plates and experiments were carried out as described above. The reactions were stopped by adding 5% trichloroacetic acid. After centrifugation of the reaction mixtures, the acid-soluble fractions were neutralized by extracting residual trichloroacetic acid with water-saturated ether and then concentrated by a freeze-dry method. For nucleoside and nucleobase analyses, aliquots of the concentrated acid-soluble fractions were injected onto a column of Cosmosil 5C18 (Nacalai Tesque, Kyoto, Japan) equilibrated with 0.02 M KH_2PO_4 (pH 5.6). The column was eluted with a linear gradient of 0–40% methanol (flow rate, 1.0 mL/min), with monitoring at 254 nm. Identification of each peak was achieved by the procedures described by Hartwick *et al.* [10]. For nucleotide analyses, aliquots of the concentrated acid-soluble fractions were separated by polyethyleneimine cellulose TLC as described by Henderson *et al.* [11].

Measurement of hypoxanthine transport. PAEC (1×10^4 cells/cm^2) were seeded into 6-well tissue culture plates, and experiments were carried out as described for [^{14}C]hypoxanthine metabolism except for a labeling time of 5 sec instead of 60 min to avoid further metabolism of hypoxanthine [12]. We confirmed that the hypoxanthine transport proceeded linearly up to 5 sec under these experimental conditions.

Measurements of PRPP synthetase activity, PRPP level and hypoxanthine-guanine phosphoribosyltransferase (HGPRT) activity in PAEC

PAEC were cultured in 25-cm^2 flasks until semi-confluence. Then the medium was replaced with DMEM supplemented with 10% dialyzed FBS plus the addition(s) indicated, and the cells were incubated for 24 hr. PRPP synthetase was assayed essentially by the procedure of Roth *et al.* [13]. For measurement of the PRPP content, the cells were washed with phosphate-buffered saline, and PRPP was extracted with 0.2 mL of 50 mM Tris–HCl, 1 mM EDTA (pH 7.4) at 100° for 1 min. PRPP was measured essentially by the procedure of Gordon *et al.* [14]. For measurement of HGPRT activity, the cells were detached from the flasks with 0.25% trypsin, 0.02% EDTA, washed, resuspended in HGPRT enzyme buffer, and then freeze-thawed. HGPRT activity was measured, as described [15], by conversion of [^{14}C]hypoxanthine (0.02 μCi/mmol, 60 μM) into [^{14}C]IMP in the presence of excess PRPP.

Measurement of purine de novo *synthesis*

The rates of *de novo* purine synthesis were estimated in cell suspensions by measuring the incorporation of [^{14}C]formate into cellular purines. PAEC (1.0 to 1.5×10^6 cells/25-cm^2 flask) were washed and incubated in 2 mL of glutamine-free DMEM for 30 min. Then the cells were treated with 0.5 mM [^{14}C]formate (2 μCi) and 2 mM L-glutamine, and incubated for an additional 60 min. After incubation, the cells were washed with phosphate-buffered saline twice. An $HClO_4$ extract was obtained by adding 1.5 N perchloric acid to the cell pellet, and the resulting $HClO_4$ extracts were heated at 100° for 60 min prior to isolation of total purines as purine–silver complexes [16]. The purine–silver precipitates were then washed, solubilized and counted for radioactivity with a liquid scintillation counter.

Statistical analysis

Student's *t*-test was used to determine the significance of the differences between groups.

Table 1. Effects of purines and pyrimidines on the FGF-stimulated growth of PAEC

	Cells/well ($\times 10^4$)		
	None	FGFa	FGFb
10% Dialyzed FBS	1.78 ± 0.69	1.67 ± 0.01	2.49 ± 0.06
10% FBS	10.86 ± 0.19*	11.50 ± 0.20*	14.65 ± 0.22*
Purines			
Adenine	10.02 ± 0.06*	15.21 ± 0.10*	14.53 ± 0.38*
Adenosine	9.47 ± 0.15*	15.68 ± 0.63*	13.81 ± 0.34*
Guanine	4.98 ± 0.21*	9.26 ± 0.28*	9.12 ± 0.24*
Guanosine	5.33 ± 0.17*	9.67 ± 0.24*	9.56 ± 0.38*
Hypoxanthine	6.64 ± 1.94*	16.49 ± 0.37*	15.13 ± 0.13*
Inosine	8.57 ± 0.09*	16.21 ± 0.16*	15.51 ± 0.14*
Xanthine	2.46 ± 0.08	0.74 ± 0.02*	3.22 ± 0.17
Xanthosine	2.00 ± 0.02	1.64 ± 0.04	2.22 ± 0.06
Pyrimidines			
Cytosine	1.78 ± 0.01	0.50 ± 0.01*	1.69 ± 0.01
Cytidine	1.93 ± 0.01	1.68 ± 0.01	2.15 ± 0.09
Thymine	1.92 ± 0.01	1.66 ± 0.01	2.08 ± 0.09
Thymidine	1.81 ± 0.01	1.61 ± 0.01	1.84 ± 0.02
Uracil	1.97 ± 0.01	1.66 ± 0.02	2.76 ± 0.17
Uridine	1.60 ± 0.02	0.35 ± 0.01*	1.49 ± 0.01

Cells (1×10^4 cells/2 cm^2) in 24-well (Falcon) tissue culture plates were incubated with 0.5 mL of medium containing 10% FBS or 10% dialyzed FBS in the presence or absence of 10 ng/mL FGFb or 10 ng/mL FGFa plus 10 μg/mL heparin. At this point, each compound indicated was added at a concentration of 50 μM. After 4 days of incubation at 37° in a CO_2-incubator, the cells were counted by the colorimetric MTT method as described under Materials and Methods. Each value is the mean ± SEM for three samples.

* $P < 0.01$, relative to the value observed in 10% dialyzed serum.

RESULTS

Effects of purine and pyrimidine metabolites on the limited growth of PAEC in the presence of FGFb or FGFa plus heparin

Recently, we showed that hypoxanthine in FBS is essential for both basal and FGF-stimulated growth of PAEC in FBS-containing medium [5–7]. In the present study we examined whether other purines or pyrimidine metabolites exhibited activity similar to that of hypoxanthine on the FGF-stimulated growth of PAEC. As shown in Table 1, adenine, adenosine and inosine exhibited the same strong potency as hypoxanthine, while guanine and guanosine exhibited mild activity toward the basal and FGF-dependent growth of PAEC. In contrast, neither xanthine, xanthosine, nor any of the pyrimidine metabolites had activity. However, xanthine, cytosine and uridine inhibited the stimulation of PAEC growth induced by FGFa plus heparin but not that by FGFb. This indicates that the basal and FGF-dependent growth of PAEC demand the metabolism of specific purine nucleosides.

[^{14}C]*Hypoxanthine metabolism in PAEC treated with or without FGF*

Figure 1 shows the concentration–response curves for pulse incorporation (1 hr) of [^{14}C]hypoxanthine into the acid-soluble and -insoluble fractions of PAEC, which were pretreated with or without various doses of FGFb or FGFa plus heparin for 24 hr. In control cells, about 8% of the total incorporated radioactivity was derived from the acid-insoluble nucleic acid. The addition of FGFs (1–30 ng/mL) to PAEC concentration-dependently increased the radioactivity in the acid-soluble and -insoluble fractions by about 1.5- and 2-fold, respectively. These concentration-dependencies of FGFs as to [^{14}C]hypoxanthine metabolism coincide with those of growth-promoting activity.

In many eukaryotic cell types, hypoxanthine is rapidly salvaged in conjunction with PRPP to yield IMP, which is then converted to adenine or guanine nucleotides through salvage pathways. Table 2 shows the distribution of [^{14}C]radioactivity incorporated into nucleobases, nucleosides and nucleotides in the acid-soluble fraction extracted from cells pretreated with or without FGF for 24 hr following incubation with [^{14}C]hypoxanthine for 1 hr. In control cells, radioactivity derived from [^{14}C]hypoxanthine was incorporated mainly into IMP, adenine nucleotides, uric acid and nucleic acids. In contrast, upon pretreatment with FGFa plus heparin or FGFb for 24 hr, radioactivities were increased in the ATP, ADP, GTP, xanthine and uric acid fractions. The radioactivity of IMP was decreased in FGF-stimulated cells. These results indicate that FGFs stimulate a salvage pathway for purine nucleotide synthesis involving hypoxanthine as a substrate.

PRPP is used as a substrate for both *de novo*

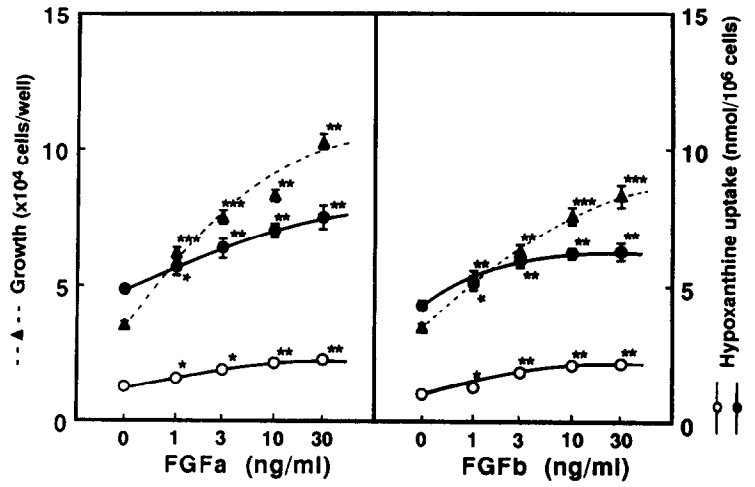

Fig. 1. Effects of FGFa plus heparin or FGFb on growth rate and [^{14}C]hypoxanthine incorporation into acid-soluble or -insoluble fraction of PAEC. PAEC (1×10^4 cells/cm^2) were seeded into 24-well tissue culture plates. In the growth rate experiments (▲), the cells were incubated for 44 hr in 0.5 mL of DMEM containing 10% FBS (hypoxanthine concentration of FBS, 105 μM) with various concentrations of FGFa plus 10 μg/mL heparin or FGFb, and then the cell numbers (▲) were determined as described under Materials and Methods. In the [^{14}C]hypoxanthine incorporation experiment (●, ○), the cells were preincubated for 12 hr in DMEM supplemented with 10% dialyzed FBS. Various concentrations of FGFa plus 10 μg/mL heparin or FGFb were then added and the cells were further incubated for 24 hr. The cells were labeled with [^{14}C]hypoxanthine (0.1 μCi; final concentration, 10 μM, in 0.4 mL DMEM) at 37° for 60 min. Then the reactions were stopped with 0.5 mL of cold 0.6 M perchloric acid. The acid-soluble (●) and -insoluble (○) fractions were separated and counted as described under Materials and Methods. Each value is the mean ± SEM for three samples. When not shown, the SEM was smaller than the size of the symbol employed. Key: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, relative to value observed in the absence of FGF.

Table 2. Effect of FGF on hypoxanthine salvage pathways in PAEC

	Radioactivity incorporated from [^{14}C]hypoxanthine (nmol/10^6 cells)		
	None	FGFa	FGFb
Total	6.78 ± 0.23	12.03 ± 0.34*	10.42 ± 0.22*
Acid-insoluble	0.54 ± 0.03	1.46 ± 0.11†	0.82 ± 0.06†
Acid-soluble			
ATP	0.61 ± 0.04	3.60 ± 0.12*	3.43 ± 0.04*
ADP	1.23 ± 0.01	1.83 ± 0.02†	1.87 ± 0.01†
AMP	0.26 ± 0.01	0.59 ± 0.02†	0.36 ± 0.02
IMP	2.07 ± 0.21	0.82 ± 0.03†	0.78 ± 0.03†
Hyp	0.47 ± 0.04	0.40 ± 0.03	0.41 ± 0.03
Xan	0.18 ± 0.01	0.52 ± 0.03†	0.35 ± 0.03†
Uric acid	0.59 ± 0.04	1.41 ± 0.15†	1.09 ± 0.12†
GTP	0.05 ± 0.002	0.88 ± 0.02*	0.67 ± 0.02*
Others	0.77 ± 0.22	0.54 ± 0.10	0.83 ± 0.07

PAEC (1×10^4 cells/cm^2) were seeded into 6-well tissue culture plates, incubated in 2 mL of DMEM supplemented with 10% dialyzed FBS in the presence or absence of 10 ng/mL FGFb or 10 ng/mL FGFa plus 10 μg/mL heparin for 24 hr, and then labeled with 0.1 μCi of [^{14}C]hypoxanthine (final concentration, 6 μM, in 1 mL DMEM) for 60 min. The reactions were stopped by the addition of 0.5 mL of 5% trichloroacetic acid. The acid-soluble and -insoluble fractions were separated and analyzed by reverse phase HPLC and TLC as described under Materials and Methods. Each value is the mean ± SEM for three samples.

* $P < 0.001$, relative to value observed in the absence of FGF.

† $P < 0.01$, relative to value observed in the absence of FGF.

Table 3. Effects of FGF on PRPP content, PRPP synthetase activity, HGPRT activity and hypoxanthine transport

	PRPP content (pmol/10^6 cells)	PRPP synthetase (μU/10^6 cells)	HGPRT activity (mU/10^6 cells)	Hypoxanthine transport (nmol/10^6 cells/min)
None	346 ± 22	5.1 ± 0.13	2.96 ± 0.28	0.122 ± 0.007
FGFa	1160 ± 28*	51.4 ± 4.7*	3.49 ± 0.35	0.141 ± 0.004
FGFb	854 ± 17*	42.3 ± 2.8*	ND†	0.129 ± 0.016

PAEC were cultured in 25-cm^2 flasks (PRPP content, PRPP synthetase and HGPRT activity) or 6-well tissue culture plates (hypoxanthine transport). The medium was then replaced with DMEM supplemented with 10% dialyzed FBS. At this point, 10 ng/mL FGFa plus 10 μg/mL heparin or 10 ng/mL FGFb was added. The cells were incubated for 24 hr. Measurement of the PRPP content, HGPRT activity and hypoxanthine transport was carried out as described under Materials and Methods. Each value is the mean ± SEM for three samples.

* $P < 0.01$, relative to value observed in the absence of FGF.

† Not determined.

synthesis and salvage pathways. Subsequently, limitation of PRPP supplementation may regulate purine synthesis. Table 3 shows the effects of FGF on PRPP content, PRPP synthetase activity, HGPRT activity and hypoxanthine transport in PAEC. The stimulation of PAEC by both FGFa and FGFb increased the PRPP content and PRPP synthetase activity 2- to 3-fold and 8- to 10-fold, respectively, but it did not enhance the activity of either HGPRT or hypoxanthine transport.

Purine synthesis de novo *in PAEC*

Next we examined the role of *de novo* synthesis of purines in the metabolism of PAEC in response to FGF. Methotrexate, a strong inhibitor of purine synthesis *de novo*, exhibited no inhibitory activity toward the growth of PAEC in medium containing 10% dialyzed FBS in the presence or absence of hypoxanthine, and even with 10% nondialyzed FBS (Fig. 2). This indicates that the *de novo* pathway may not be preferential for purine synthesis in PAEC. Therefore, we assayed the rate of incorporation of [^{14}C]formate into total purine metabolites in FGF-treated or non-treated PAEC; fibroblasts, in which *de novo* synthesis is readily measurable, were used for comparison (Table 4). FGFs were found not to change the *de novo* synthesis of purines, and the total amount of purines synthesized *de novo* in PAEC (0.144 ± 0.013 nmol/10^6 cells/hr) was much less than that in fibroblasts (0.567 ± 0.023 nmol/10^6 cells/hr); neither value was affected significantly by FGFa or FGFb stimulation.

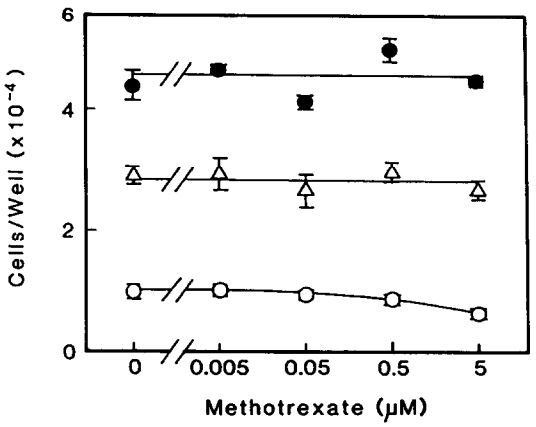

Fig. 2. Effect of methotrexate on the growth of PAEC. Cells (1×10^4/2 cm^2) in 24-well (Falcon) tissue culture plates were incubated with 0.5 mL of medium containing 10% FBS (●) or 10% dialyzed FBS with (△) or without (○) 50 μM hypoxanthine. Then, various concentrations of methotrexate were added. After 4 days of incubation at 37° in a CO_2-incubator, the cells were counted by the colorimetric MTT method as described under Materials and Methods. Each value is the mean ± SEM for three samples.

DISCUSSION

The purpose of the present study was to determine how hypoxanthine acts as an indispensable factor in the maintenance of FGF-dependent growth in PAEC. One hypothesis is that hypoxanthine may be utilized as a substrate required for FGF-stimulated nucleic acid synthesis in PAEC. In mammalian cells, hypoxanthine is known to be incorporated via purine-specific transport and then quickly metabolized through phosphoribosylation to form IMP. FGF was reported to trigger this purine-specific transport in G_0-arrested Balb/c-3T3 fibroblasts [17]. However, such FGF action was not observed in the present case of PAEC (Table 3).

Purine nucleotides of mammalian cells may be formed via *de novo* synthesis and salvage pathways. In both pathways the common substrate is PRPP, and the rate-limiting reaction is PRPP synthesis, which is formed from ribose-5-phosphate through the action of PRPP synthetase. FGF markedly enhanced PRPP synthetase activity, resulting in the accumulation of PRPP in PAEC (Table 3). A similar increase in the PRPP level was reported previously in FGF-stimulated Swiss 3T3 cells [18]. PRPP is subsequently conjugated with nucleobases to yield nucleotides, the bases of which are derived through two pathways, via *de novo* synthesis and salvage after catabolism of nucleic acids. In PAEC the former *de novo* pathway is not preferential for

Table 4. Effect of FGF treatment on [^{14}C]purine *de novo* synthesis from [^{14}C]-formate

Cells	Radioactivity incorporated into [^{14}C]purine metabolites (nmol/10^6 cells/hr)		
	None	FGFa	FGFb
PAEC	0.144 ± 0.013	0.163 ± 0.014	0.179 ± 0.014
Fibroblasts	0.567 ± 0.023*	0.622 ± 0.042*	0.596 ± 0.036*

PAEC (1.0 to 1.5 × 10^6 cells/25-cm^2 flask) were incubated with 0.5 mM [^{14}C]-formate (2 μCi) and 2 mM L-glutamine for 60 min. After incubation, the cells were extracted with $HClO_4$, and then the radioactivity incorporated into purine metabolites was measured as described under Materials and Methods. Each value is the mean ± SEM for three samples.

*$P < 0.01$, PAEC vs fibroblasts.

purine synthesis, since methotrexate did not inhibit the synthesis of nucleobases, nucleosides, nucleotides or nucleic acids. In fact, it was found that PAEC had an extremely low capacity of [^{14}C]purine *de novo* synthesis from [^{14}C]formate as compared with fibroblasts; neither value was affected significantly by FGFa or FGFb stimulation (Table 4).

Hypoxanthine is known to be metabolized mainly into nucleosides and nucleotides via the salvage pathway in PAEC (Table 2). Hypoxanthine is first converted to IMP by HGPRT, and then to adenine or guanine nucleotides. In fact, [^{14}C]hypoxanthine-loaded PAEC have high levels of [^{14}C]IMP > [^{14}C]-ADP > [^{14}C]ATP. However, the level of [^{14}C]IMP did not increase, but rather decreased in FGF-stimulated cells, in contrast to the increased levels of [^{14}C]adenine nucleotides and [^{14}C]guanine nucleotides (Table 2). It is inferred that the decrease in [^{14}C]IMP and the increases in ATP and GTP pools indicate the conversion of IMP into AMP, GMP, and their corresponding diphosphates and triphosphates, because conversion of IMP to AMP or GMP proceeds via a two-step pathway catalyzed by adenylosuccinate synthetase and adenylosuccinate lyase, or by IMP dehydrogenase and GMP synthase, respectively.

In contrast to purine bases, pyrimidine bases had little effect on the basal or FGF-dependent growth of PAEC (Table 1). This suggests that pyrimidine synthesis of PAEC preferentially depends on pyrimidine synthesis *de novo*. Although xanthine, cytosine and uridine inhibited the FGFa- but not the FGFb-stimulated growth of PAEC, further investigation is required to clarify the conflicting result between acidic and basic FGF.

Most purine compounds had some effect on PAEC growth, but there was marked diversity. Among these purines, guanine and guanosine activities were much less than those of adenine, adenosine and hypoxanthine. Such diversity between adenine bases and guanine bases in the cell-proliferating activity of a variety of purines is hard to explain, but one possibility is that it may be due to the strict regulation of the intracellular ratio of ATP and GTP. In human T lymphocytes, adenine is converted mainly into adenine nucleotides, while guanine is converted at similar rates into adenine and guanine nucleotides. Hypoxanthine is first converted into IMP, and then into either adenine or guanine nucleotides, in a ratio of about 3:1 [15]. In PAEC treated with or without FGF, five to ten times as much hypoxanthine was converted into adenine nucleotides as was converted into guanine nucleotides (Table 2). Moreover, XMP, a salvage product of xanthine or xanthosine, is converted mainly into guanine nucleotides [19]. Hence, the addition of guanine, xanthine or their nucleosides to culture medium may cause the accumulation of intracellular GTP, which is said to be inhibitory for cell growth in a mature T-cell line or B-lymphoblast line [20].

Purine nucleotides are also formed through purine synthesis *de novo*, and in this reaction PRPP is a key substrate. But the contribution of the increase in the PRPP level caused by FGFs to purine synthesis *de novo* seems to be very little because a sufficient dose of methotrexate had no effect on the growth of PAEC, even in the absence of extracellular purine compouds (Fig. 2). An increased PRPP level but no change in the rate of purine synthesis have also been demonstrated when HGPRT-deficient lymphoblast cells were incubated with inosine [21]. These data demonstrated that the dependency of FGF-stimulated growth of PAEC was due to the activation of the purine metabolite salvage pathway by FGF and the insufficient purine nucleotide synthesis via the *de novo* pathway.

We could not detect changes in any kind of known second messengers such as cAMP formation, intracellular Ca^{2+} level and arachidonic acid release in PAEC in medium containing 10% dialyzed FBS, when they were treated with hypoxanthine and other purine derivatives (data not shown). These observations indicate that hypoxanthine and other purine derivatives would not act via intracellular messenger production to enhance the FGF activity on PAEC growth.

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