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Interleukin-1β inhibits luteinizing hormone-induced plasminogen activator activity in rat preovulatory follicles *in vitro*

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The effects of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) on ovulation-associated plasminogen activator (PA) activity were investigated using preovulatory follicles excised 48 h after equine chorionic gonadotrophin (16IU)-priming of immature rats. Follicles were incubated for 6 and 14 h with a single dose of LH (1 μg/ml) only, or various cytokine doses in the presence or absence of LH. PA activity in follicular homogenates was determined by a radioactively labelled fibrin-coated plate method and secreted levels of the ovulatory mediators progesterone (P) and prostaglandin E (PGE) were measured by radioimmunoassay. LH induced time-dependent rises in PA (2.5-fold over control at 6 h and fourfold over control at 14 h), while IL-1β and TNFα alone had no effect over either time period. LH and cytokine coincubations over 14 h revealed that IL-1β dose-dependently inhibited the LH-induced increase in PA activity, up to 85%. The effects of TNFα on LH-induced PA activity were not significant. Both IL-1β and TNFα increased P and PGE secretion time- and dose-dependently. In summary, IL-1β dose-dependently inhibits the LH-induced increase in PA activity in rat preovulatory follicles *in vitro* while, as with TNFα, increasing P and PGE synthesis. This study, shows that the earlier reported pro-ovulatory action of IL-1β is not likely to be mediated by activation of the PA-system and suggests that IL-1β may mediate a regulatory loop controlling the extent and distribution of LH-induced PA activity in rat preovulatory follicles.

Keywords: rat; preovulatory follicle; plasminogen activator; interleukin-1 beta; tumour necrosis factor alpha

Introduction

Ovulation involves the cyclic degradation of follicular tissue and is temporarily correlated with an increase in plasminogen activator (PA) activity (Beers *et al.*, 1975). PA is a serine protease that catalyzes the activation of plasmin from its zymogen plasminogen. The PA system, is comprised of two activators: tissue-type PA (tPA) and urokinase-type PA (uPA), as well as two PA inhibitors: PAI-1 and PAI-2 (Danø *et al.*, 1985). The PA system is thought to promote follicular rupture via procollagenase activation by plasmin (Eeckhout & Vaes, 1977), which generates collagenase and ultimately leads to collagen breakdown in the preovulatory follicular wall and extrusion of the oocyte. Since the early studies, a specific tPA activity increase has been demonstrated at ovulation in the rat (Liu *et al.*, 1991), while the administration of serine protease inhibitors (Reich *et al.*, 1985a) or antibodies to either tPA or plasmin (Tsafriri *et al.*, 1989) suppresses ovulation. Furthermore, a directed proteolysis on the apical part of the follicle in the rat was recently indicated by findings of PA activity specifically on the protruding part of the ovulatory follicle (Peng *et al.*, 1993).

Ovulation resembles an immune reaction (Espey, 1980) and involves inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNFα) (Brännström & Norman, 1993). The presence of IL-1β mRNA in rat and human ovaries (Hurwitz *et al.*, 1991, 1992) and TNFα mRNA in rat and mouse ovaries (Sancho-Tello *et al.*, 1992; Chen *et al.*, 1993) supports a functional role for these two pluripotent cytokines in this organ, as does immunoreactive IL-1β in mouse and human ovaries (Wang & Norman, 1992; Simon *et al.*, 1994) and immunoreactive TNF detectable in human, rat and bovine ovaries (Roby & Terranova, 1989; Roby *et al.*, 1990). Factors specifically implicating important roles for these cytokines in ovulation include the presence of IL-1 and TNFα bioactivity in the perfusate of the ovulating rat ovary (Brännström *et al.*, 1994) and the ability of exogenously administered IL-1β and TNFα to significantly increase the LH-induced ovulation rate of the perfused rat ovary (Brännström *et al.*, 1992; 1993a). Reports of a preovulatory increase in IL-1β mRNA in rat thecal/interstitial cells (Hurwitz *et al.*, 1991) and a periovulatory increase in TNFα bioactivity in bovine follicular fluid (Zolti *et al.*, 1990) provide further evidence of participation of these cytokines in ovulation.

There has however been no attempt to investigate any possible link between the IL-1β/TNFα and PA systems in the ovary although the regulation of the PA response in non-ovarian systems is well known to be at least partially governed by the presence of IL-1β and TNFα (Hannocks *et al.*, 1992; So *et al.*, 1992). This latter observation raises the possibility of a role for ovarian IL-1β and TNFα in the paracrine control of PA activity at ovulation. Hence, the objective of this study was to further investigate the biochemical aspects of ovulation by assessing the effects of IL-1β and TNFα on the PA activity of whole preovulatory follicles explanted from primed rats and incubated *in vitro*.

Results

Morphological observations

Cross sections of dissected follicles revealed a well defined 4–5 cell thick theca layer with negligible

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stroma attachment. Immunohistochemical labelling with antibody MCA-149 (Figure 1A), recognizing a cytoplasmic epitope in neutrophilic granulocytes (Billet *et al.*, 1984), and antibody ED_1 (Figure 1B), specific for a cell surface determinant on monocyte/macrophages (Dijkstra *et al.*, 1985), showed the presence of these cells exclusively in the thecal layer and mainly concentrated near the basement membrane separating the thecal and granulosa layers. These leukocytes are potential sources of and responders to IL-1β and TNFα. There was an approximately fivefold excess of neutrophils (54.38 ± 5.33 stained cells/section; $n=8$ sections), compared to monocytes/macrophages (10.77 ± 1.75; $n=11$). Negative controls for ED1 and MCA 149 binding revealed 0.42 ± 0.27 ($n=6$) and 0.25 ± 0.25 ($n=4$) cells non-specifically stained, respectively.

PA activity

Neither IL-1β (0.1–10 ng/ml) (Figure 2A), nor TNFα (1–100ng/ml) alone at 14 h (Figure 2B), nor IL-1β (10 ng/ml) nor TNFα (100 ng/ml) alone at 6 h (data not shown) had any effect on follicular PA activity. LH (1 μg/ml) increased PA activity 2.5-fold and fourfold at 6 and 14 h, respectively. When IL-1β was combined with LH (1 μg/ml) over 14 h, there was a dose-dependent suppression of PA activity, with a 85% reduction in LH-induced PA activity with 10 ng/ml of IL-1β. TNFα (100 ng/ml) caused a 35% reduction in LH-induced PA activity at 14 h of incubation. Although the latter result was not statistically significant, TNFα (100 ng/ml) significantly reduced PA activity over TNFα (1 ng/ml) when either dose was coincubated with the gonadotrophin. Following the shorter incubation period of 6 h, no significant cytokine-induced inhibition of LH stimulated PA activity was seen.

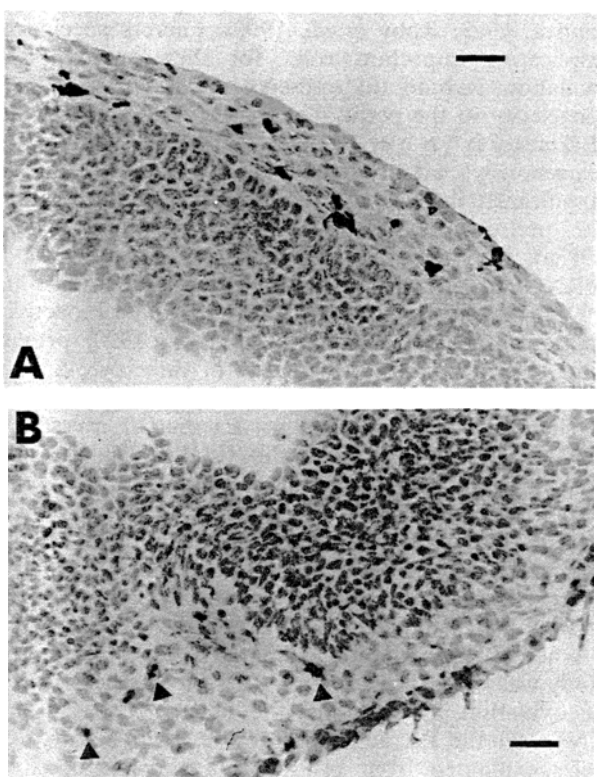

Figure 1 Immunohistochemical localization of neutrophils and monocyte/macrophages in follicle wall sections of isolated rat preovulatory follicles. (**A**) Follicle wall section incubated with MCA 149 mAb specific for neutrophils. MCA 149+ cells (with darkly stained cytoplasms) are shown to be located in the thecal layer. (**B**) Follicle wall section incubated with ED1 mAb specific for monocytes and newly migrated macrophages. ED1+ cells (darkly stained and shown by arrowheads) are also found localized to the thecal layer. Bar = 40 μm

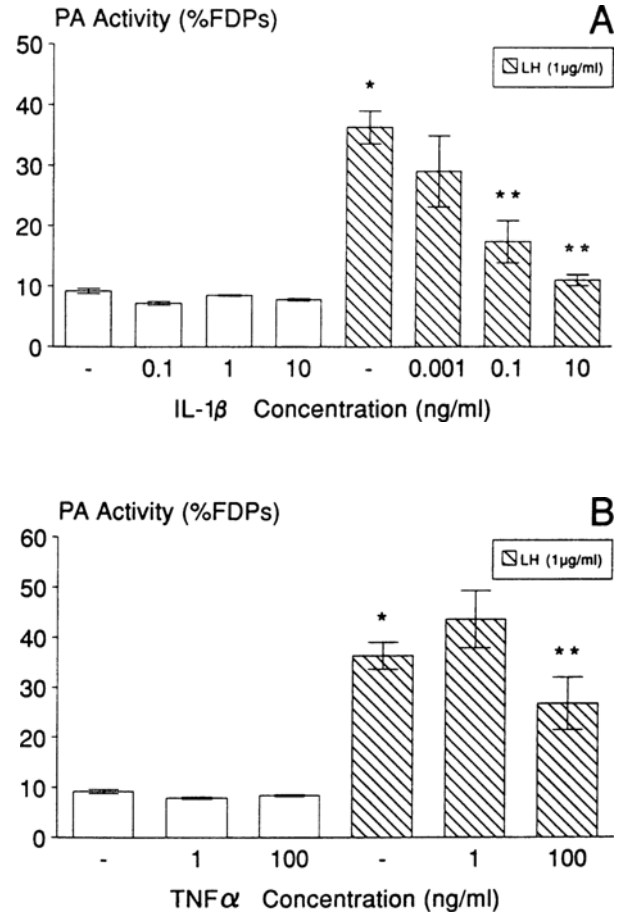

Figure 2 Effects of LH alone and (**A**) IL-1β ± LH, or (**B**) TNFα ± LH on net PA activity (Mean ± SEM), expressed as % of trypsin solubilization of fibrin degradation products (FDPs) in the homogenates of isolated rat preovulatory follicles incubated for 14 h duration. (**A**) Significance levels: *($P<0.01$) from the Control group, **($P<0.01$) from the LH group. (**B**) Significance levels: *($P<0.01$) from the Control group, **($P<0.05$) from the LH + TNF (1 ng/ml) group

P and PGE production

Significant rises over the control group in levels of P (49-fold; fourfold) and PGE (11-fold; eightfold) were observed when follicles were incubated with LH for 6 h and 14 h, respectively (Table 1). At 6 and 14 h of incubation, IL-1β enhanced PGE production up to fourfold and 11-fold, respectively, while rises due to TNFα were up to twofold and fivefold, respectively. TNFα and IL-1β had no effect on P production following 6 h of incubation, but after the longer incubation period, both cytokines had, with their maximal doses, raised P levels about twofold over control values.

Table 1 P and PGE levels in the medium of rat preovulatory follicles incubated for 6 or 14 h†

	P (pmol/ml)		*PGE (ng/ml)*	
Treatment group	*6 h*	*14 h*	*6 h*	*14 h*
Control	1.7±0.3	26.7±2.7	0.6±0.2	3.4±0.4
LH (1 μg/ml)	83.3±6.4**	112.0±7.1**	6.9±1.5*	25.5±1.7**
IL-1β (0.1 ng/ml)	–	33.6±5.4	–	7.7±1.9
IL-1β (1 ng/ml)	–	56.3±9.2*	–	35.0±10.5
IL-1β (10 ng/ml)	4.4±0.5	55.9±4.1*	2.3±0.2	38.0±3.7**
LH + IL-1β (0.001 ng/ml)‡	–	74.4±12.3	–	27.5±1.5
LH + IL-1β (0.1 ng/ml)‡	–	81.0±7.7	–	36.5±4.2
LH + IL-1β (10 ng/ml)‡	81.8±11.0	81.0±6.0	19.0±4.4***	61.6±7.5***
TNFα (1 ng/ml)	–	25.3±1.3	–	2.2±0.4
TNFα (100 ng/ml)	1.6±0.1	45.6±3.9*	1.1±0.1	16.1±3.2*
LH + TNFα (1 ng/ml)‡	–	113.6±8.3	–	23.5±1.7
LH + TNFα (100 ng/ml)‡	77.9±3.9	101.0±7.2	7.8±0.8	44.3±4.1***

†Data expressed as mean±SEM. ‡LH dose in each case is 1 μg/ml. */**Significantly different from the Control group: ($P<0.05$)/($P<0.01$), respectively. ***Significantly different from the LH group: ($P<0.01$).

Coincubation of LH with IL-1β resulted in a threefold rise in PGE levels after 6 h compared to LH alone, but there was no further change invoked by TNFα in the presence of LH. Following 14 h of incubation, coincubations resulted in further increased PGE levels with increasing IL-1β and TNFα dose, up to 2.5-fold and twofold over the LH group, respectively.

Discussion

Ovulation involves classical mediators of the inflammatory response (Espey, 1980). Of these, eicosanoids, kinins and histamine are considered to contribute via ovarian vasodilation and permeability increases, while PA and other implicated proteases act via weakening of the follicle wall involving collagen breakdown (Brännström & Janson, 1991). Collagenase activity is necessary for ovulation (Brännström *et al.*, 1988; Woessner *et al.*, 1989) and evidence exists demonstrating that collagenase activation occurs following that of the PA/plasmin system (Reich *et al.*, 1985b; Palotie *et al.*, 1987) and that IL-1β can promote ovarian biosynthesis of matrix metalloproteinases (Hurwitz *et al.*, 1993). Macrophages and neutrophils secrete IL-1β and TNFα and are also target cells for these cytokines to become activated and secrete several ovulation-associated factors (Nathan, 1987; Parker, 1991: Vassalli, 1992). Hence these two cytokines, shown conclusively to be present and active in the ovary and at ovulation (Hurwitz *et al.*, 1991; Chen *et al.*, 1993) are likely to originate in the *in vivo* situation, from the invading leukocytes (especially macrophages and neutrophils) associated with ovulation (Brännström *et al.*, 1993b), leukocytes resident in the ovary throughout the cycle (Hume *et al.*, 1984) or possibly being produced by somatic ovarian cells themselves (Hurwitz *et al.*, 1991).

Results from this study indicate that IL-1β regulates the activity of the ovulatory mediator PA. The PA regulation was in the form of inhibition of the LH-induced rise in PA activity by IL-1β. Additionally, this was a time influenced effect, being prevalent at 14 h of incubation, but absent at 6 h. The nature of the TNFα effects on LH-induced PA activity may indicate the presence of a biphasic response. The lack of PA effect with either cytokine incubated in the absence of LH however, implies modulation of the PA response is indirect and is possibly occurring at the level of the LH receptor or the post-LH receptor events that cause activation of PA. The first point may be of interest as IL-1 has previously been shown to significantly reduce the binding capacity of the LH receptor in FSH-stimulated granulosa cells when given a minimum of 12 h exposure (Gottschall *et al.*, 1988). TNFα has likewise been shown to inhibit FSH-directed LH receptor formation dose-dependently, and as with IL-1, the inhibitory effect is likely to be due to a reduction in the LH receptor content, with no change in binding affinity (Darbon *et al.*, 1989). Alternatively, the cytokines may be affecting PA activity indirectly via induction of PA inhibitors. IL-1 for instance, has in certain cell systems, been reported to increase PAI activity at the expense of PA activity (Bevilaqua *et al.*, 1986; Rogister *et al.*, 1990). Concomitant enhancement of PA and PAI activities, but more so of PAI, by the combination of LH and IL-1β in the present study remains possible. LH/hCG has been shown to also raise PAI-1 mRNA expression in rat granulosa and theca cells as the expected time of ovulation approaches (Liu *et al.*, 1991; Chun *et al.*, 1992), while non-ovarian studies have shown simultaneous cytokine enhancement of both activator and inhibitor mRNA levels within the PA system (Gross *et al.*, 1991). It should be realized that the fibrinolytic assay incorporated in this study measures net fibrinolytic activity of PA in the homogenized sample, which corresponds to relative biologically active tissue levels of PA and PAI in the whole follicle. Thus, a local increase in PA activity at the follicular apex, as has been demonstrated in the rat *in vivo* (Peng *et al.*, 1993), might be obscured by the more general inhibition of PA activity.

The normality of *in vitro* incubated follicles following 6 and 14 h incubation periods was indicated by the secretion profiles of P and PGE. The levels observed after each particular incubation time correlated with values obtained in a previous study which incorporated similar, but not identical, cytokine doses and incubation times (Brännström *et al.*, 1993c). The fact that the secretion of P and PGE increases due to IL-1β, while

tissue PA activity simultaneously decreases might be due to the synthesis or release of these mediators being activated through different pathways. Furthermore, prostaglandins have previously been shown to have an effect on collagenase synthesis, without affecting PA (Reich *et al.*, 1985b). Hence it remains a possibility that it is the sequential events of IL-1β causing production of prostaglandins, which in turn induce the synthesis of collagen degrading metalloproteinases, which may explain the observed IL-1β-induced synthesis of metalloproteinases in ovarian dispersates (Hurwitz *et al.*, 1993).

In conclusion, we have shown that IL-1β inhibits LH-induced PA activity in isolated preovulatory follicles of the rat in a dose- and time-dependent manner. It is likely that the mechanisms whereby IL-1β promotes ovulation as previously reported, involve, among other factors, enhanced output of PGE and P, but that the effect of this cytokine on PA activity in general, is not pro-ovulatory. Finally, it is put forward that IL-1β may be a mediator of a regulatory loop designed to regulate the intensity and site of LH-induced activation of PA at ovulation in accordance with the site specific proteolytic activity at the follicular apex which has recently been demonstrated in the rat (Peng *et al.*, 1993) and rabbit ovary (Tadakuma *et al.*, 1993).

Materials and methods

Reagents

Ovine LH (NIH LH s-25; specific activity 2.3 IU/mg) was a generous gift of NIDDK (Bethesda, MD) and the National Hormone and Pituitary Program (University of Maryland School of Medicine, Baltimore, MD). Equine CG was purchased from Intervet (Boxmeer, Holland); recombinant human IL-1β (specific activity 5×10^8 IU/mg) and recombinant human TNFα (specific activity 2×10^7 IU/mg) were from Genzyme (Boston, MA); bovine serum albumin (BSA; Fraction V) was from Boehringer-Mannheim (Mannheim, Germany); gentamicin sulfate, bovine thrombin, human plasminogen, and 3, 3'diaminobenzidine tetrahydrochloride (DAB) were from Sigma (St Louis, MO); [^{125}I]human fibrinogen (1.48 Ci/μg) and phosphate buffered saline (PBS) was from ICN Flow (Costa Mesa, CA); bovine fibrinogen came from Calbiochem (San Diego, CA) and human urokinase-type PA was provided by American Diagnostica (New York, NY). Isopentane was from BDH (Poole, UK) and Tissue-Tek OCT compound came from Miles Inc (Elkhart, IN). Mouse anti-rat monoclonal antibodies (mAb) against monocytes/macrophages (ED1) and neutrophils (MCA 149) were from Serotec (Oxford, UK). Horse-radish peroxidase-labelled sheep anti-mouse immunoglobulin (F/ab$\frac{1}{2}$ fragment; sheep anti-mouse HRP) was purchased from Amersham (Buckinghamshire, UK).

Animals, tissue preparation and immunohistochemistry

Immature female Sprague-Dawley rats (Central Animal House; University of Adelaide) were maintained under standardized environmental conditions (14 h light/10 h dark; lights on at 0700), receiving pelleted-food and water *ad libitum*. On the morning of day 26 of age, when the rats generally weighed 60–75 grams, they were injected s.c. with 16 IU eCG to induce follicular development. Forty-eight hours later, the rats were sacrificed by cervical dislocation and their ovaries removed and placed in chilled PBS (pH 7.4). Having removed the surrounding bursa and fat tissue, the 10–15 largest follicles (diameter > 0.7 mm) of each ovary were dissected free of adherent stromal tissue under a stereomicroscope using watchmaker forceps. Follicles of this size at proestrus can be considered to have escaped atresia and hence are destined to ovulate (Osman, 1985). The dissected follicles were pooled, washed three times in PBS by drawing back and forth through a pipette and kept in PBS on ice until ready for incubation. For morphological and immunohistochemical analysis randomly chosen clean follicles were covered with OCT and frozen in isopentane in liquid nitrogen, prior to storing at −70°C. Air-dried 6 μm-thick cryostat sections were fixed at 4°C for 10 min with 96% ethanol (for ED1 staining) or 100% acetone (for MCA 149 staining), before incubating with ED1 (1:400 dilution; mouse anti-rat mAb against monocyte/macrophages), or MCA 149 (1:800 dilution; mouse anti-rat mAb against neutrophilic granulocytes), in 1% BSA in PBS, pH 7.2 (PBS-BSA) and 10% heat-inactivated normal rat serum (NRS). Sections were then incubated in sheep anti-mouse-HRP (1:100 dilution in PBS-BSA with 10% NRS) for 2–3 h at 4°C. Bound antibody was detected by incubation with DAB (0.5 mg/ml) in 0.05 M Tris-HCl (pH 7.6) containing 1% H_2O_2. Negative controls were incubated similarly, but mAb was omitted. All slides were counterstained with hematoxylin. Two independent observers (one being blind to the treatment protocol) quantified the number of positive cells by counting all stained cells within a whole section. The inter-observer difference was less than 11%.

Incubation procedure

Eight follicles/well of a 24-well plate (Linbro, Hamden, CT) were incubated for 6 or 14 h in a total volume of 500 μl of medium ± LH ± cytokine treatments. LH, IL-1β and TNFα were all prepared to the required dilution in incubation medium. The medium was M199 with Earle's salts (Gibco, Grand Island, NY) supplemented with 0.1% BSA and gentamicin sulfate (50 μg/ml). The incubations were carried out at 37°C under carbogen gas (5% CO_2/95% O_2) as this atmosphere has been shown to be able to maintain follicles in culture for at least 24 h (Roby & Terranova, 1990). Follicles were homogenized immediately following the completion of incubation. Follicular homogenates and incubation media were stored at −20°C until analysed for PA activity and P/PGE levels, respectively. Pooled follicles from 6–8 rats were used in each incubation experiment. All treatments were performed in at least triplicate and experiments repeated at least twice.

PA assay

PA activity in the eight follicles of each well was measured after homogenization in 200 μl of M199 supplemented with 0.1% Triton-X 100 in a glass-glass homogenizer and centrifugation (10 000 *g* for 5 min). The supernatant was stored at −20°C until analysed for PA activity essentially by the method of Thakur *et al.* (1990) with minor modifications. This assay utilizes the fibrinolysis activity of plasminogen activated by PA and involves coating microwell plates (96 well; Disposable Products, Adelaide, Australia) with [^{125}I]fibrinogen, by adding 20 000 c.p.m./50 μl/well of iodofibrinogen solution (^{125}I-labelled human fibrinogen in 0.25 g bovine fibrinogen/L phosphate buffer; 0.05 M; pH 7.4), followed by overnight evaporation at 37°C. Coated plates were stored at room temperature and used within 48 h. On the day of assay the plate was incubated with bovine thrombin (1 IU/100 μl PBS per well) for 1.5 h at 37°C to allow the conversion of fibrinogen to fibrin. This was followed by three washes with phosphate buffer. More than 80% of the total counts/well were recoverable at this stage upon incubation with 0.25% (w/v) trypsin solution. Plasminogen solution (150 μl; 0.4 IU/L M199), as the reaction substrate, was immediately added, followed by 50 μl of sample. Incubation at 37°C lasted

for 18 h, during which time PA in the sample (or standard) activates plasminogen, forming plasmin, which can then cleave the well's fibrin coating into fibrin degradation products (FDPs). A standard curve spanning 1–100 IU/L of uPA in M199 was incorporated in each assay to ensure a reliable dose-response existed. Upon the completion of incubation, 100 µl medium containing solubilized [^{125}I]FDPs was harvested into polystyrene tubes and the radioactivity counted. PA activity was expressed as % total solubilization of FDPs by 0.25% trypsin solution and represented graphically (Figure 2). The PA assay was shown to be plasminogen-dependent, to be unaffected by the inclusion of 0.1% Triton X-100 and to have a low background (i.e. medium alone) FDP solubilization effect. Each experiment was assayed for PA on a single microwell plate, the intra-assay CV being 10.4%.

P and PGE assays

Levels of P in the medium were analysed using a radioimmunoassay kit from Amersham (Buckinghamshire, UK). The intra- and inter-assay CVs were <5% and <7% respectively. PGE (as its methyl oxime) was measured by radioimmunoassay, as previously described (Kelly *et al.*, 1986), with intra- and inter-assay CVs of <10% and <10.7% respectively.

Data analysis

Statistical analysis of PA activity data was performed on arcsin $\sqrt{\%}$ transformed values. An IBM SAS program was used to statistically calculate overall differences between the mean values of relevant comparable treatment groups within each investigated parameter. These were analysed using an ANOVA test for single measures. When an overall difference was identified, individual means were compared using a Scheiff's test for multiple comparisons.

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