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HORMONES - CYTOKINES - SIGNALING

TGF-β_1 dissociates human proximal tubule cell growth and Na^+-H^+ exchange activity

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TGF-β_1 dissociates human proximal tubule cell growth and Na^+-H^+ exchange activity. Stimulation of proximal tubule cell (PTC) growth in a variety of physiological and pathological renal conditions is preceded by increased renal production of transforming growth factor-β_1 (TGF-β_1) and by augmented tubular sodium transport via activated sodium hydrogen exchange (NHE). Since TGF-β_1 has been shown to be an important paracrine and autocrine regulator of PTC growth, the hypothesis that TGF-β_1 modulates basal and mitogen-stimulated PTC growth via an effect on NHE activity was examined. Confluent, quiescent, human PTC were incubated for 24 hours in serum-free media containing vehicle (control) or 1 ng/ml TGF-β_1, in the presence or absence of 100 ng/ml insulin-like growth factor-1 (IGF-I). Under basal conditions, TGF-β_1 inhibited thymidine incorporation (73.5 ± 7.3% of control, $P < 0.05$), but exerted no effect on cellular protein content (97.4 ± 10.7% of control), an index of hypertrophy. There was no significant alteration of NHE activity, measured as ethylisopropylamiloride (EIPA)-sensitive H^+ efflux (2.72 ± 0.50 vs. control 3.26 ± 0.68 mmol/liter/min) or $^{22}Na^+$ influx (2.20 ± 0.23 vs. control 2.19 ± 0.19 nmol/mg protein/min). When co-incubated with IGF-I, TGF-β_1 induced significant PTC hypertrophy (116.9 ± 8.2% of control, $P < 0.05$), which was not seen with either agent alone. TGF-β_1 counteracted the stimulatory effect of IGF-I on DNA synthesis (TGF-β_1+IGF-I 103.0 ± 7.3% vs. IGF-I alone 181.2 ± 30.3% of control, $P < 0.05$), but did not affect IGF-I-stimulated EIPA-sensitive $^{22}Na^+$ influx (3.63 ± 0.63 vs. IGF-I alone 3.67 ± 0.50 nmol/mg protein/min, P = NS, both vs. control 2.19 ± 0.19 nmol/mg protein/min, $P < 0.05$). Similar results were obtained when NHE activity was measured as EIPA-sensitive H^+ efflux. Moreover, the kinetics of NHE activation by the combination of TGF-β_1 and IGF-I (involving an increase in V_{max}) were identical to that previously found for PTC exposed to IGF-I alone. The study demonstrates that TGF-β_1 elicits distinct PTC growth responses in the presence and absence of IGF-I, without modification of NHE activity. The combination of predominant PTC hypertrophy and enhanced proximal tubule Na^+ reabsorption found in many conditions that are associated with renal growth is likely to require the integrated actions of both TGF-β_1 and IGF-I.

Loss of functional renal tissue, due either to renal disease or surgical ablation, is followed by augmented proximal tubule transport and proximal tubule cell (PTC) hypertrophy [1]. Although such changes serve to compensate for reduced nephron numbers and help to maintain glomerulotubular balance in the short term, excessive stimulation of PTC growth and transport function may ultimately be maladaptive [2]. The development of therapeutic strategies aimed at limiting this hypertrophic growth response requires further knowledge of the factors that regulate, or dysregulate, this growth response.

Transforming growth factor-β (TGF-β), a multi-functional cytokine produced by tubular, interstitial and glomerular cells, is now recognized as a key player in the regulation of PTC growth [3]. Increased renal TGF-β expression has been observed in animal models of physiological and pathological renal growth, including compensatory hypertrophy [4], subtotal nephrectomy [3], adriamycin nephropathy [5] and streptozotocin-induced diabetic nephropathy [6, 7]. TGF-β over-expression has also been documented in renal tissue obtained from patients with diabetic nephropathy, focal segmental glomerulosclerosis, IgA nephropathy, human immunodeficiency virus nephropathy and chronic renal allograft rejection [3, 8]. All are conditions where the tubulointerstitial growth response is the primary predictor of kidney failure [2, 9]. *In vitro* studies in animal PTC provide specific evidence that TGF-β promotes hypertrophy, either alone [10, 11] or in combination with mitogens, such as insulin [12] and epidermal growth factor (EGF) [13]. Moreover, Sharma and colleagues [7] demonstrated that treatment of diabetic rats with neutralizing anti-TGF-β antibodies substantially reduces PTC hypertrophy.

Despite links between other growth factors and transport [14, 15], little attention has been directed towards the specific effects of TGF-β on PTC transport processes. Proximal tubule sodium-hydrogen exchange (NHE) activity and sodium reabsorption have been shown to increase prior to the onset of compensatory renal hypertrophy [16, 17], and to be potentially linked to cell growth [18, 19] and subsequent hypermetabolic cellular injury [20]. We have previously demonstrated the key role of insulin-like growth factor-1 (IGF-I) in stimulating PTC mitogenesis and NHE [14]. As TGF-β and IGF-I are likely to have integrated effects on renal growth and transport, the effects of transforming growth factor-β_1 (TGF-β_1) on human proximal tubule growth parameters and NHE activity were determined under basal and IGF-I-stimulated conditions.

Key words: intracellular pH, kidney tubules, proximal tubule physiology, microspectrofluorimetry, sodium, sodium hydrogen antiporter.

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METHODS

Patients

Segments of macroscopically and histologically normal renal cortex were obtained aseptically from adult human kidneys removed surgically because of small (<6 cm) renal adenocarcinomas ($N = 6$), pelvic transitional cell carcinoma ($N = 1$) or angiomyolipoma ($N = 1$). The average patient age was 62.3 ± 4.3 years (mean ± SEM) and the male:female ratio was 3:5. Patients were otherwise healthy and were on no medications. Informed consent was obtained prior to their operation and the use of human renal tissue for primary culture was reviewed and approved by the Royal North Shore Hospital and University of Sydney Human Medical Research Ethics Committee.

Cell culture

The method for primary culture of human PTC is described in detail elsewhere [21]. Briefly, the renal tissue was transported immediately to the laboratory and decapsulated. The cortex was dissected from the medulla, finely minced, subjected to collagenase digestion (class 2, 383 U/mg; Worthington, Freehold, NJ, USA) for 30 minutes at 37°C and passed through a 100 μm mesh. Filtered tubular fragments were resuspended in 50 ml of 45% Percoll (Pharmacia, Uppsala, Sweden) made isosmotic with double-strength Krebs-Henseleit solution (NaCl 108 mmol/liter, KCl 4.9 mmol/liter, $CaCl_4$ 2.6 mmol/liter, NaH_2PO_4 3.1 mmol/liter, $NaHCO_3$ 28 mmol/liter), and centrifuged at 20,000 rpm (48,400 g) and 4°C for 30 minutes (Beckman J2 to 21 ultracentrifuge). The lowermost tissue band, containing highly purified populations of proximal tubule fragments, was carefully removed and washed. These tubule fragments were resuspended in serum-free, antibiotic-free, hormonally-defined media, consisting of 1:1 (vol/vol) Dulbecco's modified Eagle's media and Ham's F-12 (DMEM/Ham's F-12; ICN) supplemented with 5 μg/ml human transferrin (Sigma, St. Louis, MO, USA), 5 μg/ml (0.87 μmol/liter) bovine insulin (Sigma), 0.05 μmol/liter hydrocortisone (Sigma), 10 ng/ml (1.64 nmol/liter) epidermal growth factor (Collaborative Research Inc., Bedford, MA, USA), 50 μmol/liter prostaglandin E_1 (Sigma), 50 nmol/liter selenium (Sigma) and 5 pmol/liter triiodothyronine (Sigma). The tubular fragments were plated at a density of 1.5 mg pellet/cm^2 (approximately 5000 to 7000 fragments/cm^2) in 75 cm^2 flasks (Corning, NY, USA). Media were changed every 48 hours. The cells were incubated in humidified 95% air/5% CO_2 at 37°C and were subcultured at near-confluence using a seeding density of 4000 cells/cm^2. Such cells were designated passage 1.

Cytologic examination of cytocentrifuge preparations of cultured PTC from all donors failed to reveal any evidence of cellular atypia. The growth and transport characteristics of these cells have been previously studied in this laboratory and found to reproducibly exhibit the features of PTC *in vivo*. Specifically, these cells exhibit typical morphology along with intact tight junctions and polarized transport systems, including pharmacologically distinct apical and basolateral NHE, phlorizin-inhibitable apical Na^+-glucose transport, parathyroid hormone-inhibitable apical Na^+-phosphate transport, probenecid-inhibitable vectorial organic anion transport and quinine-inhibitable vectorial organic cation transport [21].

Experimental protocol

All studies were performed on confluent, quiescent, passage 2 human PTC grown on 24-well culture plates (Nunc, Roskilde, Denmark) for growth parameter and $^{22}Na^+$ uptake measurements, or on glass coverslips for microspectrofluorimetry. Quiescence was achieved by incubation for 24 hours in basic media (DMEM/Ham's F-12 containing 5 μg/ml human transferrin). Cells were then incubated with basic media containing vehicle (control), TGF-β_1 alone (Sigma), TGF-β_1 combined with human recombinant IGF-I (Collaborative Research Inc.), or IGF-I alone. The concentration of TGF-β_1 employed for these experiments was determined by preliminary dose-response studies, which delineated the minimum concentration of TGF-β_1 required to produce a maximal anti-proliferative effect in human PTC. The IGF-I concentration used (100 ng/ml) has been previously found in this laboratory to maximally stimulate PTC growth [14]. Unless otherwise stated, incubations were for 24 hours.

Growth parameter measurements

Growth parameters were measured according to previously described methods [14]. Twenty-four hours prior to study, 0.15 MBq (4 μCi) of [methyl-^{3}H]-thymidine (37 MBq/ml, 185 GBq/mmol; Amersham) was added to each ml of control or test media that was incubated with cells. At the end of the incubation periods, cells were dislodged with 80 μl/cm^2 CR-Dispase (Collaborative Research Inc.) for 10 minutes at 37°C. Cells were then washed three times in phosphate buffered saline (PBS) at 1250 g and 4°C for five minutes. Aliquots of the cell suspensions were retained for cell counting in a standard haemocytometer and for determination of thymidine incorporation by liquid scintillation counting in a β-counter (1215 Rackbeta II; LKB Wallac, Turku, Finland).

Cellular protein content was measured in the remaining portions of cell suspensions after cell disruption by a Boy 1000 ultrasound (Rudolf Grauer, Switzerland). Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, California, USA), using bovine serum albumin (fraction V, RIA grade; Sigma) as the standard.

Measurement of sodium hydrogen exchange activity

Microspectrofluorimetry. Microspectrofluorimetric determination of NHE activity is described in detail elsewhere [14]. Briefly, confluent monolayers of PTC grown on glass coverslips were loaded with 5 μmol/liter 2′,7′-bis(2 carboxyethyl)-5 [6]-carboxyfluorescein acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR, USA) for 30 minutes at 37°C. Following removal of residual extracellular dye, the monolayers were mounted on the stage of a Nikon-Diaphot inverted microscope in a 300 μl chamber and perfused at a rate of 1.5 ml/min with pre-warmed (37°C) Na^+-containing buffer (135 mmol/liter NaCl, 4 mmol/liter KCl, 1.2 mmol/liter $CaCl_2$, 0.8 mmol/liter $MgCl_2$, 1 mmol/liter NaH_2PO_4, 5 mmol/liter D-glucose, 10 mmol/liter HEPES, pH 7.4). The cells were excited alternately at 430 nm and 490 nm. BCECF fluorescence was detected at an emission wavelength of 530 nm. After subtraction of background fluorescence values, the 490 nm/430 nm fluorescence intensity ratios were then calculated and converted into intracellular pH (pH_i) measurements using the high K^+/nigericin calibration technique.

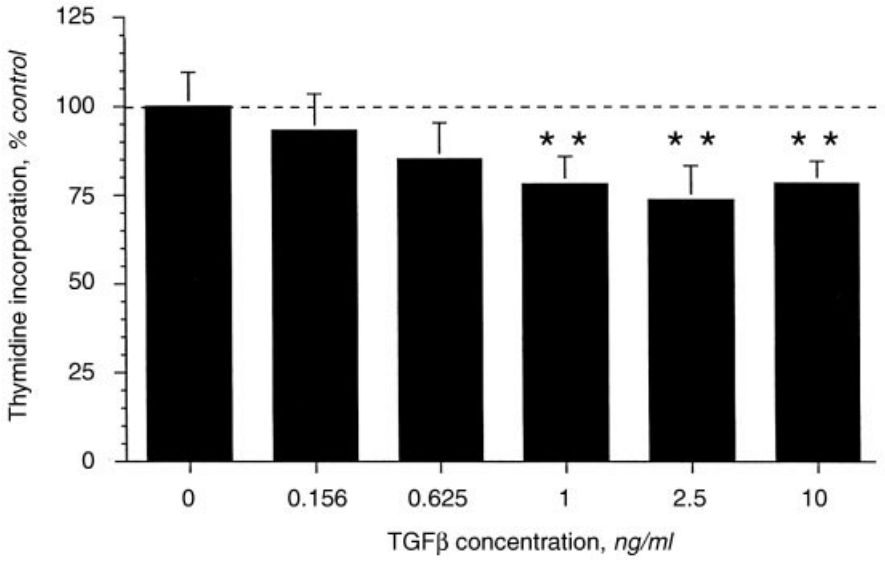

Fig. 1. Effect of transforming growth factor-B_1 (TGF-β_1) on proximal tubule cell (PTC) DNA synthesis. Thymidine incorporation was measured in confluent, quiescent human PTC incubated for 24 hours in serum-free media containing various concentrations of TGF-β_1, as indicated. Results represent the mean ± SEM of 4 experiments, each performed in triplicate. *$P < 0.05$ versus control.

During each experiment, cells were acidified by perfusion with 20 mmol/liter NH_4Cl in Na^+-containing buffer for five minutes followed by perfusion with Na^+-free buffer (135 mmol/liter N-methyl-D-glucamine, 4 mmol/liter KCl, 1.2 mmol/liter $CaCl_2$, 0.8 mmol/liter $MgCl_2$, 1 mmol/liter NaH_2PO_4, 5 mmol/liter D-glucose, 10 mmol/liter HEPES, pH 7.4) for five minutes. The cells were then perfused with Na^+-containing buffer in the presence or absence of 10 μmol/liter EIPA. The initial rate of pH_i recovery was obtained by calculating the first order derivative of the Na^+-dependent alkalinization curve during the first 60 seconds.

Intrinsic buffer capacities (β_i) were determined separately in cells from each of the control and treatment groups by sequentially pulsing them with isosmotic, Na^+-free buffers containing 0, 5, 10 or 20 mmol/liter NH_4Cl and measuring the change in pH_i. Regression lines, relating β_i to pH_i for human PTC between pH_i 6.6 and 7.6, were generated. The intracellular buffer capacities for the PTC used in the pH_i recovery experiments were calculated by interpolation from these regression equations. H^+ efflux rates were determined by multiplying the rates of change in pH_i by the β_is at the corresponding pH_i values. NHE activities were defined as the components of H^+ efflux that were Na^+-dependent and inhibited by 10 μmol/liter EIPA. This concentration of EIPA has been previously demonstrated to produce maximal inhibition of apical NHE in human PTC under basal and mitogen-stimulated conditions [14].

$^{22}Na^+$ uptake. The method used has been described previously [14]. Cell culture medium was aspirated and the cells were then washed and incubated with 200 μl isotonic, Na^+-free NH_4Cl buffer (20 mmol/liter NH_4Cl, 115 mmol/liter choline chloride, 4 mmol/liter KCl, 1.2 mmol/liter $CaCl_2$, 0.8 mmol/liter $MgCl_2$, 5 mmol/liter D-glucose, 28.3 mmol/liter HEPES, 17.7 mmol/liter Tris base, pH 7.4) for 15 minutes at 37°C. $^{22}Na^+$ uptake was measured at 37°C by removing the Na^+-free NH_4Cl buffer and replacing it with 100 μl uptake medium (pH 7.4), containing 135 mmol/liter NaCl, 4 mmol/liter KCl, 1.2 mmol/liter $CaCl_2$, 0.8 mmol/liter $MgCl_2$, 5 mmol/liter D-glucose, 28.3 mmol/liter HEPES, 17.7 mmol/liter Tris base, 100 μmol/liter ouabain (Sigma) and 0.2 μCi carrier-free $^{22}Na^+$ (1 mCi/ml; NEN Research Products, Du Pont, Wilmington, DE, USA) in the presence or absence of 10 μmol/liter ethylisopropylamiloride (EIPA; Molecular Probes, Eugene, OR, USA). The reaction was stopped at one minute by aspirating the uptake medium and rapidly washing six times with 500 μl of ice-cold 0.1 mol/liter $MgCl_2$. Intracellular $^{22}Na^+$ was then released by lysing the cells with 250 μl of 0.2 mol/liter NaOH. Aliquots were retained for liquid scintillation counting and protein estimation. Results were expressed as nmol/mg protein/min. NHE activity was defined as the difference between $^{22}Na^+$ uptake in the presence and absence of EIPA, and has previously been demonstrated by EIPA dose-response curves to reflect predominantly apical NHE activity [14].

Statistical analysis

All studies were performed in triplicate from PTC cultures obtained from four to eight separate human donors. Results are expressed as mean ± SEM. Statistical differences between groups were assessed by one-way analysis of variance (ANOVA). Pairwise multiple comparisons were made by Fisher's protected least-significant differences test. The relationships between β_i and pH_i were determined by simple linear regression analysis. Determinations of the intracellular H^+-activation kinetics for apical NHE were made by non-linear regression analysis using the Hill equation [22]. Analyses were performed using the software packages Statview version 4.5 (Abacus Concepts Inc., Berkeley, CA, USA) and Regression version M1.23 (Blackwell Scientific Publications, Oxford, UK). *P* values less than 0.05 were considered significant.

RESULTS

Basal proximal tubule cell DNA synthesis

TGF-β_1 induced a concentration-dependent inhibition of PTC incorporation of thymidine, which reached statistical significance at 1 ng/ml (Fig. 1). The concentrations at which half-maximal and maximal inhibition of DNA synthesis occurred were 0.4 and 1 ng/ml, respectively. No changes were detected in cell numbers,

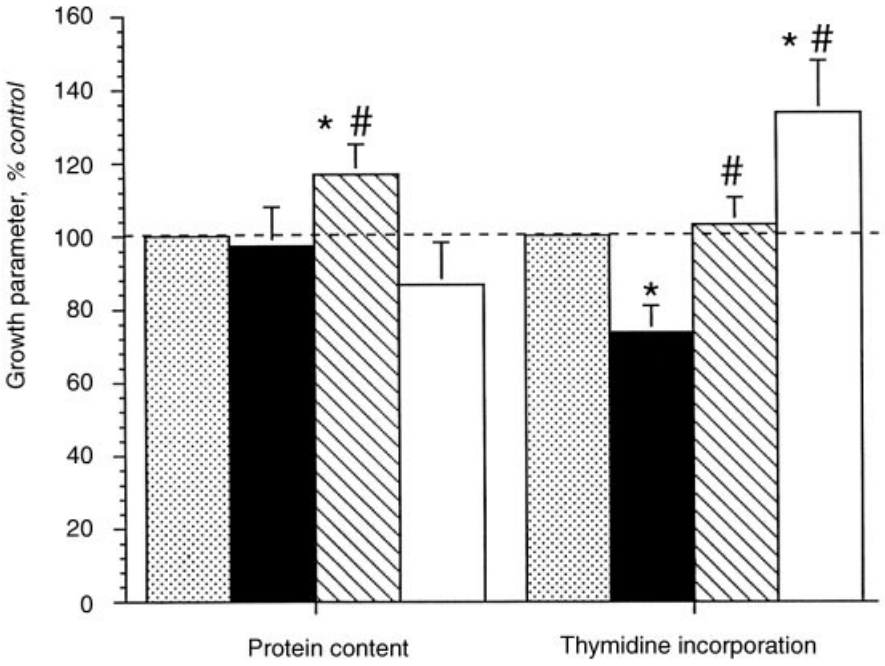

Fig. 2. Effect of transforming growth factor-B_1 (TGF-β_1) on insulin-like growth factor-1 (IGF-I)-induced human proximal tubule cell (PTC) growth. Confluent monolayers of human PTC were incubated for 24 hours in serum-free media containing either vehicle (control, ▨), 1 ng/ml TGF-β_1 (■), 1 ng/ml TGF-β_1 plus 100 ng/ml IGF-I (▨), or 100 ng/ml IGF-I (□) alone. Thymidine incorporation and cellular protein contents (expressed per cell) were measured and normalized against control values. Results represent the mean ± SEM of 8 experiments, each performed in triplicate. *$P < 0.05$ versus control. #$P < 0.05$ versus TGF-β_1 alone.

protein contents or apical NHE activities (measured as EIPA-sensitive apical $^{22}Na^+$ uptakes) at concentrations of up to 10 ng/ml (104.8 ± 6.7%, 97.4 ± 10.7% and 100.7 ± 19.9% of control values, respectively, P = NS). On the basis of these findings, a concentration of 1 ng/ml was employed for TGF-β_1 in all subsequent studies.

To exclude a time-dependent effect of TGF-β_1 on apical NHE activity, time course studies were performed in which PTC were incubated with 1 ng/ml TGF-β_1 for 24, 48 or 72 hours. Thymidine incorporation rates were significantly inhibited by TGF-β_1 at 24 hours (81.8 ± 7.9% of control values, $P < 0.05$) and 48 hours (81.5 ± 8.9% of control values, $P < 0.05$), but not at 72 hours (100.4 ± 10.1% of control values, P = NS). However, apical NHE activities were not significantly altered at any time point (98.5 ± 26.3%, 85.9 ± 28.4% and 105.9 ± 9.5% of control values, respectively, P = NS). Cellular protein contents were similarly unaffected (102.5 ± 16.9%, 101.1 ± 11.3% and 119.6%±18.4%, respectively, P = NS).

Growth response of proximal tubule cells to insulin-like growth factor-1

TGF-β_1 significantly inhibited IGF-I-stimulated PTC DNA synthesis (Fig. 2). Cellular protein content was not altered by TGF-β_1 or IGF-I alone, but was significantly increased when the two growth factors were combined (Fig. 2). PTC numbers were not significantly different between any of the study groups (TGF-β_1 alone 94.1 ± 5.3% of control, TGF-β_1 and IGF-I 93.0 ± 7.9%, IGF-I alone 109.4 ± 8.6%, P = NS).

Compared with control conditions, EIPA-sensitive H^+ efflux rates were unchanged in the presence of TGF-β_1 alone, but were significantly and comparably increased by IGF-I, both in the presence and absence of TGF-β_1 (Fig. 3). Na^+-dependent H^+ efflux was abolished by 10 μmol/liter EIPA in all groups. Kinetic studies of NHE activities with respect to intracellular [H^+] in all groups best fitted an allosteric model (Fig. 4). TGF-β_1 did not alter NHE kinetics under basal conditions. Co-incubation of

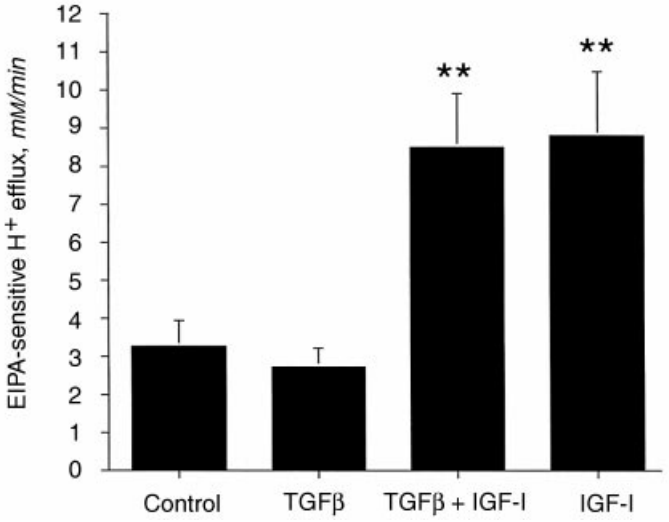

Fig. 3. Effect of transforming growth factor B_1 (TGF-β_1) on basal and insulin-like growth factor-1 (IGF-I)-stimulated sodium hydrogen exchange (NHE) activity. H^+ efflux rates following acid loading were measured in human PTC exposed for 24 hours to vehicle (control), 1 ng/ml TGF-β_1, 1 ng/ml TGF-β_1 and 100 ng/ml IGF-I, or 100 ng/ml IGF-I alone. Rates were measured in the presence and absence of 10 μmol/liter EIPA. Results represent the mean ± SEM of 7 experiments, each performed in triplicate. **$P < 0.0001$ versus control and TGF-β_1 alone.

TGF-β_1 and IGF-I resulted in a significant increase in V_{max} (16.51 ± 1.36 vs. control 8.27 ± 0.60 mmol/liter/min, $P < 0.05$) without alteration in K_m. This kinetic mode of activation is identical to that previously reported for PTC exposed to IGF-I alone [14].

When NHE activities were measured by the independent method of $^{22}Na^+$ uptake measurement, TGF-β_1 was again found to have no effect under basal conditions (2.19 ± 0.19 vs. control 2.20 ± 0.23 nmol/mg protein/min, P = NS) or following IGF-I

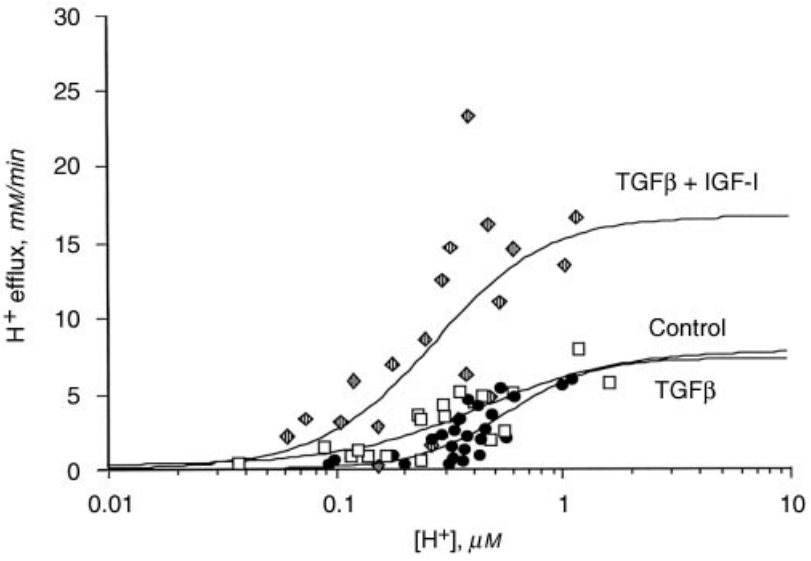

Fig. 4. Rate of H^+ efflux as a function of intracellular [H^+]. Calculated effluxes from human proximal tubule cells (PTC) following acute cell acidification were found to be dependent on intracellular [H^+] for control (□), TGF-β_1 alone (●) and TGF-β_1 + IGF-I (◇). For clarity of presentation, data from PTC exposed to IGF-I alone have been omitted, but were found to be associated with an increase in V_{max}, as described previously [14].

stimulation (3.63 ± 0.63 vs. IGF-I alone 3.67 ± 0.50, P = NS, both $P < 0.05$ vs. control). The EIPA-resistant components of $^{22}Na^+$ uptake by PTC were equivalent among all groups (control 0.71 ± 0.14, TGF-β_1 0.84 ± 0.22, TGF-β_1 and IGF-I 0.78 ± 0.14, IGF-I 0.63 ± 0.12 nmol/mg protein/day, P = NS).

DISCUSSION

The present study demonstrates that incubation of confluent, primary cultures of human PTC with TGF-β_1 significantly inhibits cellular proliferation without alteration in either cellular protein content or NHE activity. In view of the compelling evidence invoking roles for both TGF-β_1 and IGF-I in animal models of physiological and pathological renal growth [2, 3, 7, 8, 23], the combination of TGF-β_1 with IGF-I results in a distinct hypertrophic growth pattern in PTC, which contrasts with the anti-proliferative response observed with the addition of TGF-β_1 alone and the mitogenic response seen with IGF-I alone. However, TGF-β_1 modifies the growth response to IGF-I without altering IGF-I-induced activation of apical NHE. Moreover, the NHE activation kinetics for cells exposed to the combination of TGF-β_1 and IGF-I reveals a predominant increase in V_{max}, similar to that previously demonstrated for IGF-I alone [14]. Hence, the combination of TGF-β_1 and IGF-I dissociates mitogenesis and NHE activity.

Activation of NHE has been widely postulated as a possible mechanism of inducing PTC growth [16, 19]. Although most studies have focused on the link between cellular growth and the ubiquitous "housekeeping" NHE isoform (NHE1), which is found on the basolateral aspects of PTC and other epithelial cells, a recent study performed in our laboratory [14] demonstrated that apical NHE isoforms (NHE3 and/or NHE2), which represent the major entry route for sodium into PTC [14, 24], are also activated by growth factors and possibly underpin the subsequent growth response [14]. Enhanced NHE activity and augmented proximal tubular Na^+ reabsorption take place within 24 hours of a hypertrophic stimulus and prior to any demonstrable cell growth [1, 12, 16, 17]. Moreover, stimulation of NHE by a variety of mitogens is known to be a widespread phenomenon in many non-renal cells [19, 25], while blockade of NHE with amiloride analogs inhibits cellular proliferation [19, 24]. The temporal sequence and ubiquity of these events has led to the hypothesis that NHE activation transduces the signal for cellular proliferation [18, 19].

However, the converse situation, assessing whether cellular NHE activity is inhibited in the presence of anti-proliferative factors, has seldom been studied. Zaragoza, Battle-Tracy and Owen [26] argued that cellular proliferation is linked to changes in ion flux by showing that heparin engenders concentration-dependent and proportional decreases in both NHE activities and proliferation rates in vascular smooth muscle cells. However, the current report clearly demonstrates that inhibition of PTC DNA synthesis is not necessarily associated with suppression of NHE activity. This confirms a previous finding by Fine et al [12], who demonstrated that BSC-1 growth inhibitor, now considered to be identical to TGF-β_2 [27], has no effect on amiloride-sensitive $^{22}Na^+$ uptake by rabbit PTC. Unfortunately, the results of that study have been difficult to interpret because NHE measurements were performed on cells in suspension, which have subsequently been shown to poorly reflect the properties of NHE activities in adherent, polarized epithelial cells [28]. Moreover, BSC-1 growth inhibitor was found to paradoxically stimulate, rather than inhibit, PTC proliferation under basal conditions, a finding which has not been reproduced by ourselves or by other investigators [4, 10, 29, 30] using the TGF-β_1 isoform.

In the presence of IGF-I, the current study also found that TGF-β_1 could block stimulation of PTC DNA synthesis without affecting IGF-I-induced augmentation of NHE activity. Similar results have also been reported for TGF-β_2 on the effects of insulin and hydrocortisone on rabbit PTC [12], although a strong association was still observed between increased cellular protein content and augmented NHE activity. It was consequently argued that NHE activation might be a necessary requirement for renal hypertrophy, rather than hyperplasia. However, our findings that IGF-I stimulates NHE activity without altering cellular protein content indicate that PTC NHE activity can be dissociated from hypertrophy, as well as from hyperplasia. This is supported by a report by Golchini et al [31] in which NH_4Cl increased cellular

protein content in an animal proximal tubule cell line (JTC), despite concomitant suppression of NHE activity by amiloride.

The findings in the present study of inhibition of both basal and mitogen-stimulated PTC growth without alteration of NHE activity complement and extend the observations by other investigators that these processes are dissociated by selective inhibition of NHE activity. For example, Grantham and coworkers [32] noted that hexamethylene amiloride, an amiloride analog with low affinity for sodium channels and high affinity for NHE, does not limit compensatory renal hypertrophy in unilaterally nephrectomized mice. Unfortunately, the investigators did not confirm inhibition of NHE activity. Mackovic-Basic et al [33] observed that mutant LLC-PK$_1$ cells with NHE activities of less than 5% of that of control cells could still be stimulated to significantly increase their protein contents following administration of 1 μmol/liter insulin or 1 nmol/liter IGF-I. Although NHE activity was also augmented by approximately 50% in the mutant cells, the absolute activity was less than that under control conditions. Recognizing the limitations of performing studies in mutated, immortalized cell lines, subsequent studies by the same group [34] have further shown that denervation of remnant rat kidneys at the time of contralateral nephrectomy prevents stimulation of NHE activity in rat proximal tubule brush border vesicles, but does not affect either the course or extent of compensatory renal hypertrophy, as determined by measurement of kidney weight. However, the absence of an increase in NHE activity in brush border membrane vesicles, which possibly lack the full complement of cytosolic NHE-associated regulatory molecules, does not exclude NHE activation in native, intact proximal tubule cells [24, 35].

Dissociation of growth and NHE activity in human PTC in the current study also does not rule out a growth-regulatory role for sodium flux due to transport pathways other than NHE. Walsh-Reitz, Toback and Holley [36] reported that TGF-β_2 attenuated the EGF- and serum-induced increases in intracellular sodium content in BSC-1 cells. Since the amount of peptide required to inhibit growth was twice that required for inhibition of sodium influx, they argued that cell sodium content could be a necessary but insufficient signal to stimulate DNA synthesis. In addition, TGFβ_1 has been shown to inhibit Na^+,K^+-ATPase [37, 38] in rabbit proximal tubule cells and diminish Na^+-phosphate cotransport, but not Na^+-amino acid transport, in OK cells [39]. It should be noted however, that in human proximal tubule cells in the present study, EIPA-resistant Na^+ uptake was not altered by TGF-β_1. The measurements for this parameter were uniformly small though, and the method may not have been sufficiently sensitive to allow comment on Na^+ uptake by pathways other than NHE.

Interestingly, in keeping with the results of other studies [29, 30], neither TGF-β_1 nor IGF-I induced an increase in cellular protein content on their own. However, the combination of the two growth factors resulted in a significant hypertrophic response. A similar increase in cellular protein content has been reported for the combination of TGF-β_1 with other mitogens, such as insulin [12] and EGF [13]. This may be related to a TGF-β_1-induced block at the G_1/S restriction point [40] following mitogen-stimulated entry into the cell cycle. Alternatively, both IGF-I and TGF-β_1 are known to inhibit proteolysis [11, 41], such that the combination of the two may sufficiently block intracellular protein degradation to generate a measurable increase in cellular protein content.

Predominant PTC hypertrophy with minimal hyperplasia [15] and enhanced proximal tubule Na^+ reabsorption (via apical NHE) characterize many physiological and pathological renal growth states, such as compensatory hypertrophy or diabetic nephropathy [1, 17]. Our results suggest that *in vivo* renal growth, and its associated changes in proximal tubule Na^+ transport, may be caused by the integrated actions of both TGFβ_1 and IGF-I. Indeed, overexpression of both growth factors has now been documented in a large number of experimental and human kidney disorders [3, 8, 23].

In conclusion, the results of the present study suggest that altered NHE activity is not a necessary concomitant of changes in tubular cell proliferation. Moreover, the combination of predominant PTC hypertrophy and early enhanced proximal tubule Na^+ reabsorption found in many human renal pathophysiologic conditions may be linked to the integrated actions of TGF-β_1 and IGF-I.

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