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Free amino acids mimic the anabolic but not the proliferative effect of albumin in OK proximal tubular cells

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When plasma proteins leak from circulation into the renal tubular lumen in the proteinuric renal diseases, nephrotoxicity of filtered albumin (and/or molecules bound to it) may be important in the subsequent development of tubulo-interstitial damage which contributes to the progression of the disease. When cultured opossum kidney (OK) proximal tubular cells were exposed to bovine serum albumin for 3 days *in vitro*, increased cell division ([^{3}H]-thymidine incorporation) and cellular hypertrophy (increased protein/DNA ratio) were observed. Both effects were halved if defatted albumin was used. A trivial explanation for the growth responses is that free fatty acids carried on the albumin, and amino acids generated by intracellular degradation of the albumin, are exerting a non-specific growth effect as metabolic fuels which are oxidized to generate ATP. However, the water-soluble free fatty acid octanoate (1 mmol l^{-1}) had no significant effect on protein/DNA ratio and a very variable stimulatory effect on [^{3}H]-thymidine incorporation, whereas an essential amino acid mixture or 1 mmol/l^{-1} L-Ala or L-Phe only increased the protein/DNA ratio. Furthermore no carnitine was added to the culture medium. This absence would have impaired mitochondrial transport (and hence oxidation) of long-chain free fatty acids derived from the albumin. L-Phe is also a poor substrate for mitochondrial oxidation in kidney. It is therefore concluded that the growth effects of albumin in OK proximal tubular cells are specific effects of the albumin protein and of the free fatty acids and amino acids derived from it, and not a non-specific effect on metabolic fuel supply. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS — albumin; amino acids; free fatty acids; opossum; protein degradation; proteinuria; proximal tubular cells; renal tubular hypertrophy

ABBREVIATIONS — BSA, bovine serum albumin; CRF, chronic renal failure; dpm, disintegrations per minute; FFA, free fatty acid; OK, opossum kidney proximal tubular epithelial cells; MEM, Eagle's Minimum Essential Medium; PCA, perchloric acid; PD, protein degradation; PS, protein synthesis; TCA, trichloroacetic acid

INTRODUCTION

An important aim of research in nephrology is to slow the progression of chronic renal failure (CRF).[1] It is thought that several factors are involved in progression, one of which is leakage of plasma proteins[2,3] and cytokines[4] through damaged glomeruli into the proximal tubule where these proteins trigger inflammation and scarring in the tubules and associated interstitium. As much of this leaked protein is albumin, there is currently considerable interest in the effect(s) of albumin on the biology of the epithelial cells lining the lumen of the proximal tubule.[5,6]

A major effect of albumin on cultured proximal tubular cells is the stimulation of DNA synthesis and cell division.[6,7] However, a less well studied aspect of albumin action is the accompanying anabolic response, leading to cellular hypertrophy,[7] a process which has also been implicated in the progression of CRF.[8] A simple explanation not previously investigated is that intracellular degradation of albumin endocytosed through the megalin–cubulin

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receptor complex[9] in the brush border membrane of the proximal tubular cells yields free amino acids and free fatty acids (FFAs), the two major metabolic fuels of renal cortex *in vivo*.[10,11] Both growth responses could therefore be a non-specific consequence of supply (directly into the endosomal compartment of the cell) of metabolic fuels which are then oxidized in mitochondria to generate ATP.

The aims of this study were firstly to show that the hypertrophic response to albumin is a genuine anabolic response involving changes in protein metabolism, rather than an artifact arising from other forms of protein accretion, e.g. albumin binding to the outside of the cell, entrapment in the extracellular matrix or accumulation of endocytosed albumin in the endosomal compartment. An anabolic response can arise either from increased protein synthesis (PS) or decreased protein degradation (PD). Demonstrating increased PS in cells exposed to albumin is not proof of an anabolic effect, as the increased PS might be a consequence of the synthesis occurring during the cell cycle[12] because of the cell *proliferation* response to albumin. A more reliable test of whether cellular hypertrophy arises from a distinct anabolic effect is therefore to measure PD.[13] The second aim of this study was to test the idea that albumin exerts its anabolic effect on confluent proximal tubular cell cultures by a non-specific feeding effect arising from increased supply of FFAs and free amino acids as fuels. This was done by comparing the anabolic response to albumin with that obtained on supplementing the culture medium with a water-soluble FFA supplement, with individual free amino acids, or with an amino acid mixture.

METHODS

Choice of model

Experiments were performed on opossum kidney (OK) proximal tubular epithelial cells using globulin-free bovine serum albumin (BSA; Sigma A-7638) or a defatted preparation of the same material (Sigma A-0281) at $10\,mg\,ml^{-1}$ of medium, since this highly differentiated cell line has been used with BSA at this concentration previously[7] without toxic effects.

Cell culture

OK cells were seeded at passage 65 to 70 at 10×10^4 cells per well onto plastic 35-mm diameter culture wells in Growth Medium comprising Dulbecco's Modified Eagle's Medium/Nutrient Mixture F12 (1:1 vol/vol; Invitrogen 21331) supplemented with $2\,mmol\,l^{-1}$ glutamine, penicillin ($10^5\,IU\,l^{-1}$), streptomycin ($100\,mg\,l^{-1}$), and 10% vol/vol foetal bovine serum (FBS). Serum was heat-inactivated for 30 min at 56°C before use. Cultures were incubated at 37°C under humidified 95% air/5% CO_2. Fresh growth medium was added on day 3 (i.e. after 2 days) and day 5. On day 8 the confluent cultures were rinsed with Hanks' Balanced Salt Solution to remove serum and were incubated for 72 h in test media made from serum-free Eagle's Minimum Essential Medium with Earle's Salts (MEM; Invitrogen 21090) and antibiotics and glutamine as above, supplemented with albumin, fatty acids or amino acids as required, and an additional $8\,mmol\,l^{-1}$ $NaHCO_3$ to give a final pH of 7.4 under a 5% CO_2 atmosphere.

Study design

Study 1—Protein degradation. In six experiments, cells were incubated with albumin or (as an anabolic positive control) a supraphysiological concentration of bovine insulin ($100\,nmol\,l^{-1}$).

L-[U-^{14}C]-Phenylalanine (Amersham CFB 70) was added to the growth medium (on days 5 to 8 inclusive) at a final concentration of $0.23\,mCi\,l^{-1}$ ($8.5\,MBq\,l^{-1}$) to label the cellular proteins. At the end of this labelling period, cultures were incubated in 2 ml of test medium for 2 h to eliminate radioactivity from rapidly degraded proteins.[14] The medium was then discarded, and at this point (designated time 0), a further 3 ml of test medium was added. From this point onwards, test media were supplemented with unlabelled L-phenylalanine ($2\,mmol\,l^{-1}$) to minimize re-incorporation of labelled phenylalanine into cellular protein.[14] Rates of PD were measured from the rate of release of acid-soluble ^{14}C into the culture medium[14] by sampling 0.3-ml aliquots after 7, 21, 31, 48 and 72 h. The labelled medium was mixed with an equal volume of 20% w/v trichloroacetic acid and chilled at +4°C for at least 30 min to precipitate protein. The samples were then centrifuged at 3000 *g* for 10 min at +4°C and ^{14}C-Phe activity determined in the supernatant by liquid scintillation counting as an index of PD.[14] PD rates are expressed as the rate of decline of $\log_{10}$ of the percentage of the total ^{14}C (acid-soluble ^{14}C released into the medium plus total ^{14}C remaining in the cells) recovered in each culture well.[14] Rates were calculated as the linear regression slope through the six time points of the 72 h time course (including time 0).[14] The correlation coefficient was greater than 0.998 over this time course under all incubation conditions.

At the end of the incubations, the cultures were placed on ice and rinsed three times with 0.9% w/v NaCl to remove extracellular protein and ^{14}C-Phe, before storage at −20°C. Cultures were thawed at room temperature, scraped into 1.20 ml of 1.75 mol l^{-1} (10% w/w) perchloric acid (PCA), digested at 70°C for 20 min and then chilled at +4°C for at least 30 min to maximize protein precipitation. The digests were then centrifuged at 3000 *g* for 10 min at +4°C and ^{14}C-Phe activity and total DNA[15] were determined in the supernatant. The protein pellet was dissolved in 1.00 ml of 0.5 M NaOH and ^{14}C-Phe activity and total protein[16] were determined.

Study 2—Nutrient effects. In two initial experiments, cells were incubated with albumin (with or without fatty acids) to confirm the previously reported effects on cell growth.[7] Four further experiments were then performed with additional cultures to test the effect of amino acid supplements. Data from all six experiments are presented.

After 70 h of incubation with test media, DNA synthesis was determined by incubating cells for the final 2 h in test media containing [6-^{3}H]-thymidine (Amersham TRK-61) at a final concentration of 2 mCi l^{-1} (74 MBq l^{-1}) with no added unlabelled thymidine. Labelled media were then aspirated, the cells were rapidly rinsed with 1 ml of Hanks' Balanced Salt Solution (HBSS) and then incubated for 20 min in test media containing 10^{-4} mol l^{-1} unlabelled thymidine followed by rinsing rapidly on ice with 1 ml per well of 0.9% NaCl, 5 × 0.5 ml of 10% w/v trichloroacetic acid (TCA) and 0.5 ml of 0.9% NaCl. The cells were scraped into 1 ml of 1.75 mol l^{-1} (10% w/w) PCA and digested at 70°C, and radioactivity, total protein and DNA were determined as described above.

Choice of supplements

At 10 mg ml^{-1} BSA, which has 607 amino acid residues and molecular mass 70 kDa, provides about 90 mmol of amino acid residues per litre of medium. The fraction liberated as free amino acids and FFA during the study is unknown but, assuming a moderate FFA/albumin stoichiometry of 1:1[6] and as little as 1% albumin degradation, this would yield approximately 1 mmol of total amino acids or FFA per litre of medium. Supplements were therefore tested at this concentration.

To avoid detergent effects of FFA and the need for a carrier molecule such as albumin, the water-soluble short-chain FFA sodium n-octanoate (Sigma C-5038; which is known to be a metabolic fuel for cultured proximal tubular cells[17]) was used as the FFA supplement. Four amino acid supplements were tested. L-Ala was added as it is readily transaminated to pyruvate which is a direct substrate for oxidation through pyruvate dehydrogenase and the Krebs cycle in the renal cortex,[18] generating ATP (accompanied by some conversion to L-Gln[18]). L-Leu has been shown to undergo rapid oxidation in the kidney and is thought to be a significant contributor to renal ATP generation *in vivo*.[11] In contrast renal L-Phe is converted to L-Tyr *in vivo* and largely released into the circulation without further oxidation.[11] L-Phe was therefore added as a negative control for effects on ATP synthesis. Some effects of amino acid supplements on proximal tubular cells cannot be fully reproduced by adding individual amino acids.[19] The effect of a mixture (2 × MEM) was therefore tested by doubling the concentration of all the amino acids in the medium except L-Gln (i.e. the essential amino acids) leaving L-Gln constant at 2 mmol l^{-1}.

Statistical analysis

Data are presented as mean ± SEM and were analysed using the SPSS statistics package version 10.1. The *n* value denotes the number of replicate experiments, with six replicate culture wells in each. Pooled data are presented from all experiments. Statistical significance of changes was assessed by analysis of variance and Duncan's Multiple Range Test. For the data in Figure 1 with widely differing variances, Tamhane's Test was used as the *post hoc* test. Effects were regarded as significant if $p < 0.05$.

RESULTS

Growth responses to albumin

As in an earlier report,[7] exposing proximal tubular cells to albumin increased the protein content but not the DNA content of the cultures (Tables 1 and 2) leading to an apparent cellular hypertrophy (increased protein/DNA ratio) which was blunted, but not abolished, when the albumin was defatted (Tables 1 and 2). This was accompanied by a decrease in PD rate (Table 1) which was similar in magnitude to that induced by insulin, and was also blunted by defatting. Albumin therefore seemed to be exerting a potent anabolic effect on cellular protein metabolism rather than increasing cell protein by binding to (or entrapment within) the cells.

The anabolic effect was accompanied by increased DNA synthesis (Figure 1) suggesting increased cell

Table 1. Study 1—the effect of albumin and insulin on protein degradation rate in OK proximal tubular cells

$n=6$	Control	BSA (fat replete) $10\,mg\,ml^{-1}$	BSA (defatted) $10\,mg\,ml^{-1}$	Insulin $100\,nmol\,l^{-1}$
Protein degradation rate (log_{10} %/h $\times 10^3$)	7.4 ± 0.2	$6.4^* \pm 0.2$	6.9 ± 0.2	$6.1^* \pm 0.2$
Protein (μg per 35-mm well)	481 ± 12	$574^* \pm 10$	$533^* \pm 7$	$601^* \pm 11$
DNA (μg per 35-mm well)	57.6 ± 1.3	57.6 ± 0.5	56.3 ± 0.8	59.1 ± 1.0
Protein/DNA ratio ($\mu g\,\mu g^{-1}$)	8.4 ± 0.2	$10.0^* \pm 0.1$	$9.5^* \pm 0.1$	$10.2^* \pm 0.2$

*$p < 0.05$ versus control.

Table 2. Study 2—the effect of albumin, octanoate and amino acids on DNA and protein/DNA ratio in OK proximal tubular cells

$n=6$	Control	BSA (fat replete) $10\,mg\,ml^{-1}$	BSA (defatted) $10\,mg\,ml^{-1}$	Octanoate $1\,mmol\,l^{-1}$
Protein/DNA ratio ($\mu g\,\mu g^{-1}$)	8.0 ± 0.4	$10.9^* \pm 0.3$	9.1 ± 0.2	9.0 ± 0.4
DNA (μg per 35-mm well)	43.8 ± 1.9	47.9 ± 2.0	45.5 ± 1.6	42.4 ± 1.8
$n=4$	Control	L-Ala $1\,mmol\,l^{-1}$	L-Phe $1\,mmol\,l^{-1}$	L-Leu $1\,mmol\,l^{-1}$	MEM amino acids (2×)
Protein/DNA ratio ($\mu g\,\mu g^{-1}$)	7.6 ± 0.5	8.2 ± 0.3	8.2 ± 0.3	7.9 ± 0.3	8.4 ± 0.1
DNA (μg per 35-mm well)	45.2 ± 2.7	46.5 ± 2.3	46.2 ± 2.5	47.2 ± 2.4	47.4 ± 2.2

*$p < 0.05$ versus control.

division, although in only 72 h this had negligible net effect on cell number (DNA content), possibly because of a compensatory stimulation of apoptosis.[20] As shown previously[7] this response was also blunted by defatting the albumin.

Effect of nutrient supplements

Free amino acid supplements had no effect on DNA synthesis (Figure 1) whereas the water-soluble FFA octanoate increased DNA synthesis in five out of six experiments. The mean effect was large but very variable and hence did not reach statistical significance. The reason for the variation is unknown. Higher doses of octanoate ($\gg 1\,mmol\,l^{-1}$) were not tested because such doses of short-chain fatty acids decrease intracellular pH in proximal tubular cells.[21]

In contrast, free amino acids (especially the MEM mixture) exerted an anabolic effect on total cell protein (Figure 2) similar in magnitude to that of defatted albumin, with no significant accompanying effect on DNA (Table 2). Furthermore, the FFA supplement (octanoate) had no significant effect on cell protein (Figure 2) even though defatting the albumin had substantially reduced the protein content of the cultures (Figure 2), suggesting that endogenous fat-like molecules bound to albumin were exerting effects distinct from those of octanoate.

DISCUSSION

Growth effects of albumin: a model for disease progression or an artifact of feeding the cells?

In Figure 1 the FFA component of albumin was found to be important in the cell division response, whereas free amino acids mimicked the anabolic response of defatted albumin (Figure 2) possibly by suppressing the PD rate.[19] This dependence of the growth response on the type of nutrient tested suggests therefore that these are not non-specific metabolic fuel effects arising simply from supplying fuel for oxidation to generate ATP. This is confirmed by the observation that even though L-Leu and L-Phe differ widely in their ability to serve as fuels for ATP production in kidney,[11] no significant difference was found between the effectiveness of the three individual amino acids tested here (Figure 2). This contrasts with the situation in cultured skeletal muscle cells in which L-Leu[22] and L-Gln[23] were anabolic, whereas L-Ala was not.[22,23] Mimicry of other albumin effects on OK cells by free amino acids has also been reported. The downregulation of albumin endocytosis by pre-incubation with albumin is also seen on pre-incubation with L-Ala and L-Gln, but not L-Leu, L-Glu or L-Asp.[24]

In contrast to the amino acid supplements, in Figure 2 the effect of FFA on total cell protein seemed to depend on the source (and hence presumably the

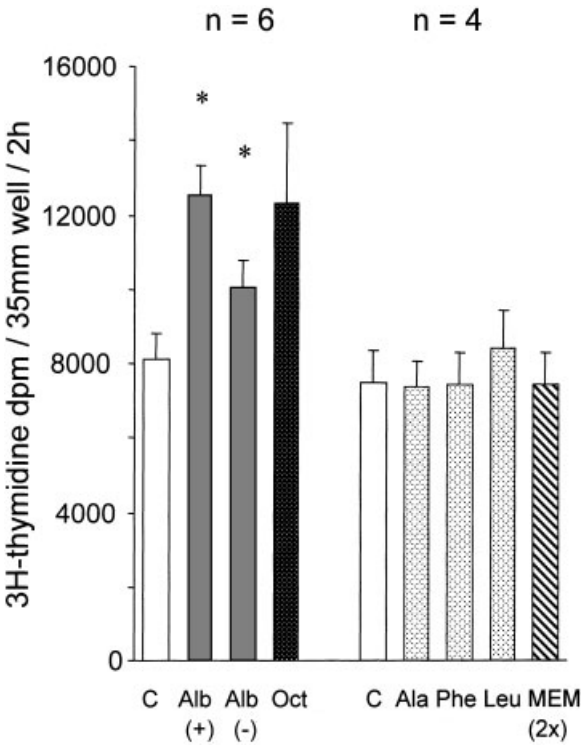

Figure 1. Study 2—effect of bovine serum albumin, free fatty acid or free amino acids on DNA synthesis rate ([^{3}H]-thymidine incorporation) in confluent OK opossum kidney proximal tubular cells. Cells were cultured for 3 days in Minimum Essential Medium with 2 mmol l^{-1} L-glutamine without serum at pH 7.4. Uptake of [^{3}H]-thymidine was determined in the last 2 h of the incubation. Incubation conditions were as follows: C, control; Alb (+), bovine serum albumin (10 mg ml^{-1}) replete with fatty acids; Alb (−), defatted bovine serum albumin (10 mg ml^{-1}); Oct, sodium n-octanoate (1 mmol l^{-1}); Ala, L-alanine (1 mmol l^{-1}); Phe, L-phenylalanine (1 mmol l^{-1}); Leu, L-leucine (1 mol l^{-1}); MEM (2 ×), MEM with 2 mmol l^{-1} L-glutamine but with all other amino acid concentrations doubled. *$p < 0.05$ versus control

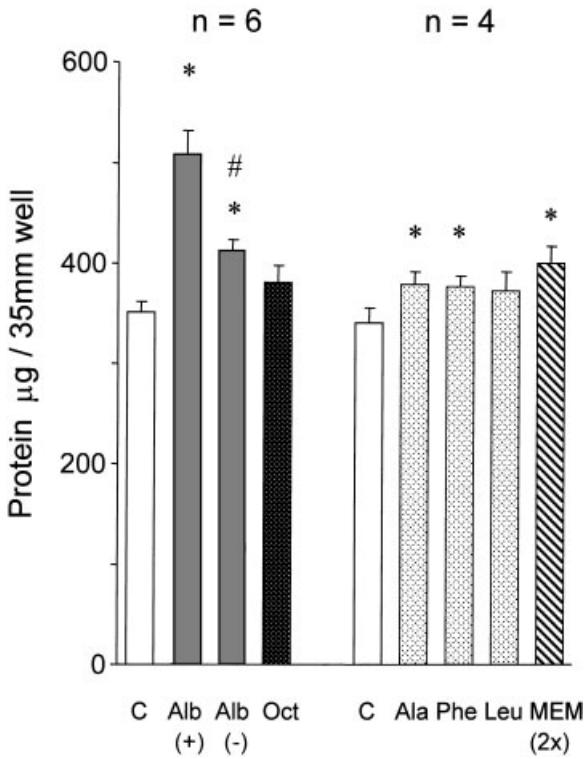

Figure 2. Study 2—effect of bovine serum albumin, free fatty acid or free amino acids on total protein content of confluent OK opossum kidney proximal tubular cells. Cells were cultured for 3 days in Minimum Essential Medium with 2 mmol l^{-1} L-glutamine without serum at pH 7.4. Incubation conditions were as follows: C, control; Alb (+), bovine serum albumin (10 mg ml^{-1}) replete with fatty acids; Alb (−), defatted bovine serum albumin (10 mg ml^{-1}); Oct, sodium n-octanoate (1 mmol l^{-1}); Ala, L-alanine (1 mmol l^{-1}); Phe, L-phenylalanine (1 mmol l^{-1}); Leu, L-leucine (1 mol l^{-1}); MEM (2 ×), MEM with 2 mmol l^{-1} L-glutamine but with all other amino acid concentrations doubled. *$p < 0.05$ versus control; $^{\#}p < 0.05$ versus Alb (+)

type) of FFA tested. Adding a high concentration of octanoate had little effect, whereas defatting (i.e. removal of the relatively small amount of FFA and other hydrophobic material attached to the albumin), markedly blunted the anabolic response to albumin. These results are consistent with a recent study on human proximal tubular cells from this laboratory in which the effects on DNA synthesis and fibronectin production of four different FFAs conjugated to albumin were found to depend strongly on the particular FFA tested.[6] It should also be emphasized that no carnitine was added to the serum-free medium used in the present study. Therefore mitochondrial transport of the endogenous long-chain FFAs carried on the albumin would probably have been a limiting factor if their non-specific oxidation as fuels were their main mode of action. Absence of carnitine has indeed been shown to block the suppression of glycolysis by a long-chain FFA supplement in cultured proximal tubular cells.[25] Even though short-chain FFA (e.g. octanoate) can enter mitochondria without carnitine, in Figure 2 octanoate still exerted less of an effect than the endogenous FFAs carried on albumin, suggesting a non-mitochondrial site of action.

Mimicry of insulin by amino acids

A potent stimulus for hypertrophic growth in cultured proximal tubular cells is transforming growth factor

beta (TGF-beta).[8] This is unlikely however to mediate the hypertrophic effect of albumin reported here (Tables 1, 2 and Figure 2) for two reasons. Firstly treatment of cultured proximal tubular cells with serum proteins causes no detectable secretion of TGF-beta.[26] Secondly, there is considerable evidence that in cultured proximal tubular cells TGF-beta induces hypertrophy by blocking progression through the cell cycle at the G1-S transition, thereby increasing cell size at the expense of cell division.[13] The growth responses observed here differed from this in that albumin stimulated both cell protein content and cell division rate (Figures 1 and 2)—an effect more reminiscent of the action of insulin. There is now much evidence that intracellular signalling pathways activated by insulin can also be activated by free amino acids,[27] but this does not fully explain the present results, as the amino acid supplements tested here only increased cell protein content (Figure 2) and not cell division (Figure 1), whereas even defatted albumin also stimulated cell division (Figure 1). This may mean that the intact defatted protein has additional biological effects not attributable to the free amino acids derived from it.

It is concluded therefore that while some but not all of the growth effects of albumin can be reproduced by adding free amino acids and (debatably) water-soluble FFA, this is not a trivial consequence of a non-specific increase in fuel supply to the cells; and implies that albumin exerts a complex but clinically significant effect on proximal tubule biology.

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