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Effect of serum albumin on vascular smooth muscle metabolism

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Abstract

In studies on metabolism of vascular smooth muscle, it was observed that incubation of intact porcine carotid artery strips with 3% bovine or porcine serum albumin had profound effects on the oxidation of substrates and O_2 consumption. Arteries incubated over 180 min with charcoal-treated and dialyzed albumin demonstrated time-dependent stimulation of glucose oxidation (145%; $P < 0.0001$, $n = 6$) and O_2 consumption (116%; $P < 0.001$, $n = 6$). These results were not mimicked by incubation with 3% solutions of ovalbumin or porcine skin gelatin. However, the oxidation of the medium chain fatty acid octanoate was inhibited in the presence of albumin over a broad range of octanoate concentrations (0.5–5.0 mM). Short chain fatty acid oxidation (acetate, 5 mM), in contrast, was not inhibited by albumin. Wash-out of albumin only partially reversed the stimulation of O_2 consumption and incubation of arteries with a polyanionic compound, polyethylene sulfonate (5 mg/ml), blunted the stimulatory effect of albumin on O_2 consumption. Albumin also produced anaplerosis of the Krebs cycle, and an increase in the content of glutamate and alanine ($P < 0.005$, $n = 8$). The metabolic effects of albumin were associated with time-dependent uptake of albumin (30.9 ± 1.5 nmol/g per 210 min; $P < 0.01$, $n = 15$). ATP-dependent proteolysis of the albumin taken up was also observed. These results demonstrate novel and important intracellular effects of serum albumin on energy metabolism of vascular smooth muscle.

Keywords: Smooth muscle; Energetics; Albumin; Metabolism

1. Introduction

In studies on metabolism of vascular smooth muscle, it had been demonstrated [1–6] that preparations of intact arterial segments could easily oxidize fatty acids that are of short or medium chain length (acetate or octanoate) and that oxidation of these substrates increases when the arteries are stimulated to contract with a contractile agonist [1,2]. However, although the short and medium chain fatty acids are readily oxidized by smooth muscle, long chain fatty acids such as palmitate (16 carbons) are the most abundant and present in the serum in the highest concentration [5,6]. Palmitate oxidation had been measured in resting segments of rabbit or rat aorta incubated within metabolic vials [4,7–9] but not in preparations of arterial segments subjected to resting tone and not in arteries stimulated to contract and generate contractile force. The relationship of palmitate metabolism with that of glucose, the other major oxidative substrate, also has not been determined. To perform such studies and to simulate physiological conditions, the incubation medium containing palmitate should also contain serum albumin because pal-

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mitate is ordinarily present in the serum complexed to albumin. Furthermore, palmitate is not readily soluble in physiological salt solutions unless complexed to albumin. Accordingly, the purpose of this investigation was to measure the oxidation of palmitate in segments of resting porcine carotid arteries and in arteries subject to contractile stimulation. However, throughout the course of these investigations, it was observed that serum albumin alone, independent of fatty acid, had pronounced effects on the oxidative metabolism of the tissue. Therefore, in this paper, we describe the alterations in metabolism of porcine carotid artery produced by albumin. Following the report by Schnitzer and Bravos [10] that isolated cultured cells of the arterial wall can take up and metabolize albumin, we demonstrate that uptake and degradation of albumin takes place in the intact tissue as well. Evidence is presented suggesting that albumin taken up exerts important metabolic effects.

2. Materials and methods

Porcine carotid arteries were procured from the slaughterhouse within 15–20 min of killing. Helical strips were prepared and mounted in a jacketed organ bath and attached to force transducers to monitor force. In each experiment four strips prepared from four different animals were mounted in the organ bath. Passive stretch was applied to the strips to simulate 100 mm Hg mean arterial pressure [11]. Preparation of the helical strips resulted in functional denudation of the endothelial cell layer as evidenced by their inability to relax in response to acetylcholine after contraction by norepinephrine. The arterial strips were incubated for 1 h at 37°C in oxygenated medium (95% O_2/5% CO_2) which consisted of 118 mM NaCl, 20 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.6 mM $CaCl_2$ and 5.6 mM glucose. After this 1 h pre-equilibration period, the medium was switched to one consisting of the same electrolyte composition but with addition of 0.5 mM palmitate complexed with 3% essentially fatty acid-free bovine serum albumin (Sigma). Preparation and dialysis of palmitate–albumin-containing medium was as described by Crass et al. [12] resulting in 1 mol palmitate bound/mol albumin. This medium contained either 150 μCi/100 ml of [9,10-^{3}H]-palmitate or 100 μCi/100 ml [6-^{3}H(N)]glucose. In other experiments 100 μCi/100 ml of either [8-^{3}H]octanoate or [^{3}H]acetate was used as the isotopic tracer. The production of ^3H_2O from these isotopes corresponded to oxidation of the respective substrates [1]. In other experiments, palmitate was omitted from the medium and only albumin was present. For estimates of glycolysis 40 μCi/100 ml of [5-^{3}H]glucose was used [2,13]. In other experiments, glucose, palmitate, and albumin were all deleted.

Oxygenation of the carotid strips was accomplished by circulating the palmitate–albumin medium through a membrane oxygenator (Radnoti Glass Technology, Monrovia, CA). The organ bath was modified so that the perfusate entered through a port at the bottom of the chamber and exited at the top of the organ bath to circulate through the membrane oxygenator with the aid of a peristaltic pump. The total perfusate volume was approximately 107 ml. Four carotid arterial strips each weighing ~0.5–0.75 g wet weight were mounted in the organ bath.

2.1. Substrate oxidation

At various times, 0.5 ml aliquots of incubation medium were withdrawn from the organ bath for quantitation of ^3H_2O production as an estimate of oxidation of ^{3}H-labeled substrates. The ^3H_2O was separated from the labeled, unmetabolized substrate remaining in the incubation medium by column anion exchange chromatography, as previously described in detail [1] using Bio-Rad AG1X8 anion exchange resin. At the end of experimentation, the arteries were immediately removed, blotted and weighed.

2.2. Oxygen consumption

The consumption of O_2 was measured separately utilizing a sealed organ bath (31.5 ml volume) in which was embedded an O_2 electrode [14]. Palmitate–albumin medium, previously oxygenated by circulation through the membrane oxygenator, was introduced from a reservoir connected to a port into the organ bath. The medium was changed with fresh

oxygenated medium approximately every 15 min and O_2 consumption was determined from the decline in O_2 tension in the bath with time. After termination of experimentation the membrane oxygenator, tubing and organ bath were circulated with 1 N NaOH followed by distilled H_2O to eliminate residual albumin and contaminating bacteria.

2.3. Perchloric acid extracts

At the end of experimentation the strips, after weighing, were rapidly frozen in liquid nitrogen, pulverized, and extracted with 60% perchloric acid ($\sim$10% of the wet weight of the muscle). Preparation of perchloric acid extracts was as previously described [15]. The following metabolites present in the extracts were assayed enzymatically as described in detail [15]: citrate, oxaloacetate, malate, α-ketoglutarate, pyruvate, aspartate, alanine, glutamate.

2.4. Lipid extraction and thin layer chromatography (TLC)

Frozen carotid strips were pulverized and extracted with 15 ml/g tissue of chloroform/methanol (2:1 v/v) overnight at room temperature at ambient conditions. The insoluble cellular debris was removed and the Folch procedure [16] was used to extract and separate the lipid and aqueous phases. Total radioactivity in each phase was determined and the chloroform was evaporated from the lipid phase by passing a stream of N_2 over the sample.

The concentrated tissue sample was plated on TLC plates (which were penetrated with fluorescein stain) and developed in the first dimension to separate polar from neutral lipids in a solution of chloroform:methanol:H_2O (65:25:4). To separate the neutral lipid classes, the plates were developed in the second dimension in petroleum:petroleum ether:acetic acid (80:20:1) [16]. Standards of the lipid classes (Sigma) were chromatographed simultaneously with the tissue sample to ascertain the identity of the chromatographed spots. The spots were detected by UV light, scraped, eluted in 1 ml of heptane:isopropanol:1 N sulfuric acid (10:40:1) and counted in liquid scintillant.

2.5. Uptake and degradation of albumin

Arteries were incubated in medium containing 3% albumin and 2 μCi/100 ml ^{14}C-methylated albumin. At 30 and 210 min, the albumin was washed out of the extracellular space by eight rinses (60 ml each) of albumin-free medium, after which time the arteries were removed from the organ bath, blotted, weighed, and immersed in 2 ml of a 2% solution of sodium dodecyl sulfate (SDS) to solubilize the muscles. After treatment for 48 h with constant stirring under ambient conditions, aliquots of muscle extract were counted for radioactivity. To determine the extent of intracellular degradation of albumin, the arteries were first frozen in liquid nitrogen and pulverized to a fine powder. The tissue powder was extracted with 3 ml of 5% trichloroacetic acid (TCA). This was centrifuged at 15 000 $\times g$ for 15 min; the radioactivity in 1 ml aliquots of supernatant was measured to determine the quantity of TCA-soluble products of albumin hydrolysis. The precipitated pellet was resuspended and solubilized in 3 ml of 2% SDS-0.25N Na_2HPO_4 after stirring for 24 h. After centrifugation, aliquots of supernatant were counted for radioactivity to determine the TCA-insoluble but SDS-soluble incorporation of albumin into the arteries.

In other experiments, carotid arteries were incubated for 1 h in normal medium (no albumin) containing 100 μCi/100 ml of [U-^{14}C]glucose to label the tissue glutamate carbon pool [17,18]. Subsequently, the [U-^{14}C]glucose was washed out of the extracellular space by eight rinses with radioactive-free medium and the incubation continued in the presence and absence of 3% albumin for an additional 210 min. Perchloric acid extracts of the arteries were prepared as described above and aliquots of extract were applied to TLC plates; the TLC plates were developed in a solution of ethanol (95%):H_2O (77:33) to separate tissue amino acids. Standards of amino acids were chromatographed simultaneously with the tissue samples and the spots were detected by spraying the chromatograms with ninhydrin reagent. The spots were scraped, extracted with H_2O for 1 h and counted for radioactivity. The following R_f values for the major tissue amino acids were determined: glutamate, 0.75; aspartate, 0.68; alanine, 0.61.

2.6. Reagents

All chemicals and enzymes was purchased from Sigma Chemical Co. [6-^{3}H(N)]Glucose and [U-^{14}C]-glucose were purchased from NEN; [9,10-^{3}H]-palmitate and ^{14}C-methylated albumin (^{14}C-methylated lysine residues) were purchased from Sigma; [5-^{3}H]glucose was purchased from Amersham; [^{3}H]-acetate was purchased from ICN; [8-^{3}H]octanoate was purchased from American RadioLabeled Chemicals (St. Louis, MO).

2.7. Statistics

Statistical significance of differences in metabolic rates over time and between different experimental conditions was determined using repeated measures ANOVA. Student's *t*-test was used when comparing means of two separate groups with equal variances. One way ANOVA followed by a Newman–Keuls multiple comparison test was used in assessing differences of three or more groups. A *t*-test for paired comparisons was used in comparing the change in means within the same group. Unless otherwise specified, *n* refers to number of experiments; there were four different arteries from different animals in each organ bath in each experiment. $P < 0.05$ was considered significant.

3. Results

3.1. Palmitate oxidation

Fig. 1 shows the time course of oxidation of palmitate in resting carotid artery strips subjected to passive stretch simulating a mean arterial pressure of 100 mM Hg. The concentration of palmitate used in these experiments was 0.5 mM. The oxidation of palmitate in resting muscles was linear with time (linear regression coefficient, $r = 0.978$) and was 0.17 ± 0.03 nmol/min/g ($n = 10$) (or 0.17 ± 0.07 nmol/min/g, 95% confidence interval). Contractile activation of the muscles upon challenge with 100 μM norepinephrine was not accompanied by augmented oxidation of palmitate. Single control experiments were performed to ascertain whether the experimental conditions for the determination of oxidation of palmitate were optimal. In one experiment, palmitate was present at a concentration of 1.0 mM; in another experiment, 1.0 mM carnitine was included in the incubation. In neither experiment was the rate of palmitate oxidation appreciably different (0.16 nmol/min/g and 0.13 nmol/min/g, respectively) from that measured under the previously described condition; i.e., the values for palmitate oxidation rate fell within the 95% confidence interval of the mean value.

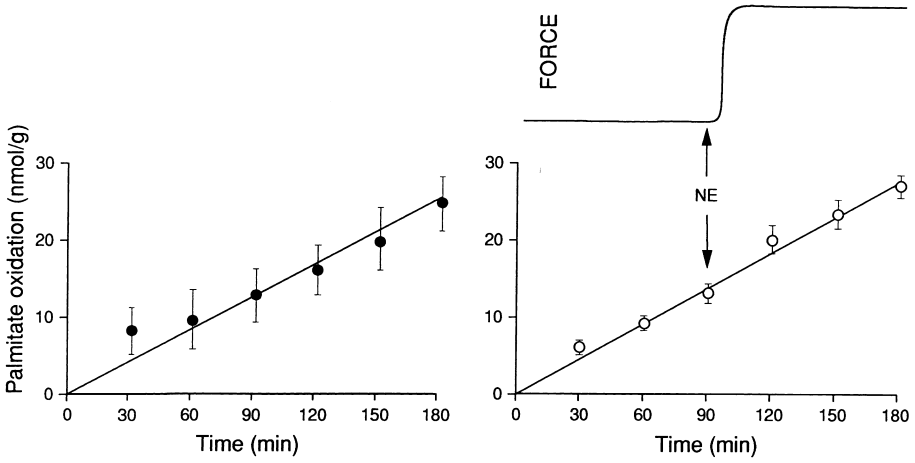

Fig. 1. Oxidation of palmitate with time in resting porcine carotid artery. Incubation medium contained glucose+palmitate–albumin. (Left panel) Palmitate oxidation in resting carotid strips ($n = 10$). (Right panel) Palmitate oxidation before and after challenge with 100 μM norepinephrine (NE). NE was added after 90 min (arrow) and the arteries remained contracted for the additional 90 min ($n = 6$). The upper panel shows a schematic diagram of force recording. There was no change in palmitate oxidation upon NE contraction. Points represent means ± S.E.M.

The incorporation and distribution of [^{3}H]palmitate into the tissue lipids was investigated. In these experiments, after 180 min of incubation with [^{3}H]palmitate the radioactive palmitate was washed out of the extracellular space by repeated rinses with fresh non-radioactive medium, after which time the arteries were frozen and the lipids extracted. Total palmitate incorporation into tissue lipids was 0.13 ± 0.06 μmol/g ($n = 6$). Only a trivial quantity of ^{3}H-labeled was detected in the aqueous phase of the lipid extraction solution (0.0008 ± 0.0002 μmol/g). The aqueous phase contains the water-soluble tissue metabolites, such as tissue free amino acids and intermediates of the Krebs cycle. Exogenous [^{3}H]palmitate incorporated into all major cellular lipid classes. In two experiments, the distribution of the ^{3}H label in the lipid classes separated by thin layer chromatography was as follows (% total label incorporated): phospholipid, 59.7%; glycerides, 4.3%; cholesterol, 1.6%; free fatty acid, 30%; unknown, 4.4%.

3.2. Glucose oxidation

Fig. 2 shows the time course of oxidation of glucose in resting carotid strips incubated with medium containing palmitate and albumin. Inspection of the time course of glucose oxidation suggested that the rate of glucose oxidation increased with time. Statistical analysis confirmed that there was a significant increase in glucose oxidation rate with time (repeated measures ANOVA, $P < 0.0001$). By the end of 180 min of incubation, palmitate–albumin stimulated the rate of glucose oxidation by 145%; 4.46 ± 0.49 μmol/g glucose per 180 min was oxidized in the presence of albumin as compared to 2.11 ± 0.05 μmol/g per 180 min in the absence of glucose (or 4.46 ± 1.26 μmol/g vs. 2.11 ± 0.12 μmol/g; 95% confidence interval).

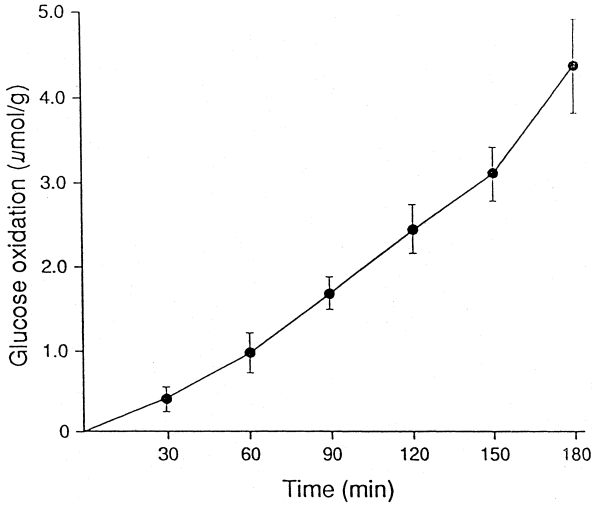

Fig. 2. Oxidation of glucose with time in resting porcine carotid artery. Incubation medium contained glucose+palmitate–albumin. Points represent mean ± S.E.M. The rate of glucose oxidation increased with time ($P < 0.0001$, $n = 6$).

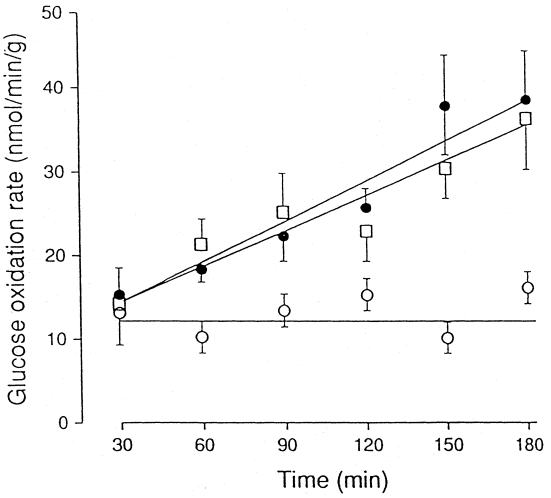

Fig. 3. Change in rate of oxidation of glucose in resting artery in the presence or absence of palmitate, albumin, or both. Incubation medium contained glucose only (○); glucose+palmitate–albumin (●); or glucose+albumin alone (□). Points represent average rates of glucose oxidation over the preceding 30 min ± S.E.M. Glucose oxidation rate increased with time in medium containing palmitate–albumin or albumin alone. With glucose alone, the glucose oxidation rate was constant ($P < 0.0001$, $n = 6$, vs. either palmitate–albumin or albumin alone).

Whether the increasing rate of glucose oxidation was attributable to an effect of palmitate and albumin, alone or in combination, was determined in a series of experiments in which glucose oxidation was measured in medium containing albumin alone (free albumin). The medium was prepared identically except palmitate was omitted. As may be seen in Fig. 3, there was no difference in the pattern of glucose oxidation when palmitate was deleted. Hence, the effect of enhanced glucose oxidation was apparently mediated by free albumin alone.

Other control experiments were performed in which the albumin was treated with charcoal and then filtered and dialyzed so that possible contami-

nant metabolites (such as other fatty acids, amino acids) bound or complexed with the albumin could be eliminated or minimized according to the method of Chen [19]. The fatty acid content of the charcoal-treated and dialyzed serum albumin was determined according to the colorimetric method of Lauwerys [20]. No fatty acid was detected in the albumin when quantities as large as 30–50 mg of protein were assayed. Furthermore, when palmitate was added to fatty acid-free albumin samples at a molar ratio of palmitate to albumin of 1:1, and the fatty acid–albumin complex was dialyzed for a day, 97% of the added palmitate was recovered. These data indicate that the metabolic effects of albumin were not caused by fatty acid contamination in the albumin. Despite this treatment of the albumin, glucose oxidation (5.48 μmol/g by 180 min) was still enhanced and to the same degree as without treatment. Two preparations of bovine serum albumin, one prepared by heat fractionation and one prepared by ethanol fractionation, were also individually tested; no difference was found between the two preparations (3.93 μmol/g and 3.72 μmol/g glucose oxidized per 180 min, respectively). We also tested fatty acid-free pig serum albumin; it had the same stimulatory effect as did bovine serum albumin (3.2 μmol/g glucose oxidized per 180 min). Similarly, to ascertain whether the apparent effects of mammalian serum albumin could be replicated with other protein solutions, glucose oxidation was measured in protein solutions (3 g/100 ml) containing gelatin from porcine skin (75 or 175 Bloom) or chicken egg albumin (ovalbumin). No stimulation of glucose oxidation was observed with these protein solutions (1.9 μmol/g and 2.1 μmol/g glucose oxidized per 180 min, respectively). Another control experiment was conducted to exclude the possibility that bacterial contamination of the albumin-containing incubation medium was responsible for the observed enhancement of glucose oxidation. Streptomycin (100 mg/l), a broad spectrum antibiotic, was added to the incubation medium. Antibiotic treatment did not negate the stimulatory effect of albumin on glucose oxidation (4.26 μmol/g glucose oxidized per 180 min). Furthermore, circulation of 1 N NaOH solution (to kill any bacteria that may be present) before the experiment did not eliminate the enhancing effect on glucose oxidation. (Note that all of the above values for control experiments for glucose oxidation fell within the 95% confidence interval of the respective mean value.)

Having established that glucose oxidation was stimulated by free albumin alone, the question arose whether the stimulation of glucose oxidation was attributed to a simple effect of albumin to stimulate increased glycolytic flux. Accordingly, the rate of glycolysis was measured in the presence and absence of free albumin. In contrast to the case of oxidation of glucose, the rate of glycolysis remained constant during the 180 min period of experimentation, in either the presence or absence of albumin (0.096 ± 0.003 vs. 0.102 ± 0.003μmol/min/g; $n = 4$, NS). Thus, the stimulation of glucose oxidation by free albumin was not attributed to a stimulating effect on glycolysis. However, this finding did not rule out the possibility that a greater portion of the total glycolytic flux was channeled to oxidative metabolism, as compared to the flux of aerobic glycolysis represented by lactic acid production. Accordingly, lactate production was measured in the presence and absence of albumin. In the absence of albumin (control), the rate of lactate production was 0.16 ± 0.02 μmol/min/g ($n = 6$) over the first 90 min of incubation; it remained unchanged over the next 90 min of incubation (0.16 ± 0.01 μmol/min/g, $n = 6$). In the presence of albumin, by contrast, lactate production over the first 90 min was 0.16 ± 0.01 μmol/min/g but it decreased significantly over the next 90 min period to 0.13 ± 0.01 μmol/min/g ($n = 4$, $P < 0.02$, paired t-test). Thus, lactate production decreased as glucose oxidation was increasing (Fig. 2).

3.3. Oxygen consumption

The consumption of O_2 was measured in porcine carotid strips incubated in medium without palmitate–albumin and was found to remain constant throughout the period of experimentation (Fig. 4). O_2 consumption was also measured over time in resting carotid strips incubated in medium containing either palmitate–albumin or free albumin alone (Fig. 4). The rate of the consumption of O_2 increased linearly with time under both substrate conditions; there was no difference in the magnitude of stimulation of O_2 consumption between the two substrate conditions. A control experiment was conducted in albumin-free medium containing 0.1 μg/100 ml

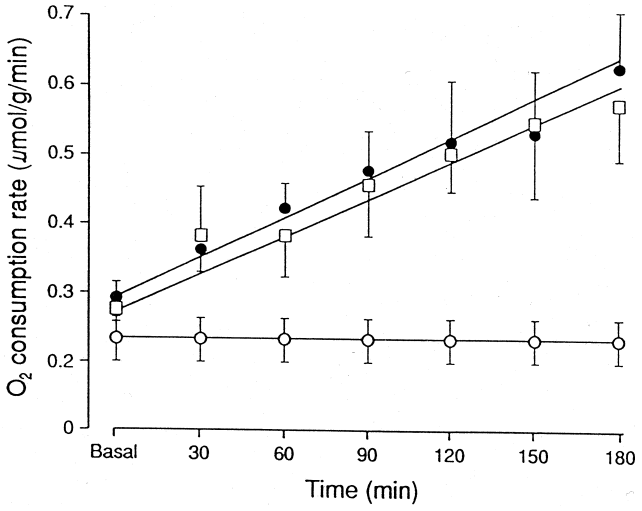

Fig. 4. Change in the rate of O_2 consumption with time in resting porcine carotid artery. Incubation medium contained: glucose alone (○); glucose+palmitate–albumin (●) or glucose+albumin alone (□). The rate of O_2 consumption increased with time with albumin alone; palmitate had no effect on O_2 consumption. In the absence of albumin O_2 consumption remained constant. Points represent average rates of O_2 consumption over the preceding 30 min ± S.E.M. ($n=4$).

L-thyroxine to exclude the possibility that thyroid hormone was a contaminant in the albumin preparation causing the stimulation of O_2 uptake; no augmentation (0%) of O_2 consumption could be demonstrated. Therefore, it appears that, as was the case with glucose oxidation, free albumin alone had a stimulating effect on the rate of O_2 consumption.

To ascertain whether the effect of free albumin on O_2 consumption was reversible, a control experiment was undertaken wherein resting arteries were incubated with albumin for 180 min after which time the albumin was washed out of the organ bath by several rinses of fresh albumin-free medium. The bath was then replaced with medium not containing albumin and O_2 consumption was measured 90 min after initial wash-out of the albumin. Basal O_2 consumption was 0.195 μmol/min/g; incubation with free albumin resulted in an increase of O_2 consumption to 0.587 μmol/min/g (~200% increase in O_2 consumption from the basal rate). After the additional 90 min incubation in medium without albumin, O_2 consumption decreased to 0.338 μmol/min/g but was still elevated ~73% above the basal, pre-albumin rate. This experiment was repeated with porcine serum albumin (3%). Basal O_2 consumption (in the absence of albumin) was 0.167 μmol/g/min. By the end of 180 min of incubation with albumin the rate of O_2 consumption increased to 0.373 μmol/g/min or a 123% increase. After 90 min repeated rinses with fresh medium without albumin, the rate of O_2 consumption decreased to 0.26 μmol/g/min but it was still elevated ~56% above the basal resting rate. These results indicate that the effect of albumin is only partially reversible, at least over the time period tested.

Whether the stimulation of O_2 consumption by albumin was attributed to stimulation of mitochondrial activity and not to other cellular O_2-consuming processes (such as peroxidation reactions) was determined by measuring albumin stimulation of O_2 consumption in the presence of sodium azide (NaN_3). Azide is a specific inhibitor of mitochondrial electron transport. When the arteries were incubated for 1 h with 10 mM NaN_3, O_2 consumption in the absence of albumin decreased from the basal rate by −40 ± 8% ($n=3$); upon incubation of the arteries for an additional 180 min in medium containing NaN_3 and albumin, O_2 consumption continued to decline by −55+5% ($n=3$) below the basal rate

Table 1
Inhibition of albumin-induced stimulation of O_2 consumption

Condition	Change from basal rate (%)		n
	60 min (albumin absent)	180 min (albumin present)	
Control	0	+115 ± 18	11
NaN_3	−40 ± 8*	−55 ± 5*	3
PES	0	+36 ± 9*	3

O_2 consumption was measured in the presence or absence of either 10 mM sodium azide (NaN_3) or 5 mg/ml polyethylene sulfonate (PES) for 60 min, after which time the incubation continued for an additional 180 min but with albumin included. Values represent mean ± S.E.M. percent change from basal rate before additions of drugs or albumin. n = different experiments, each utilizing four different arteries from different animals. *$P<0.05$ when compared with control.

Table 2
Effect of albumin on intermediates of the Krebs cycle and subsidiary transaminase reactions

Condition	Citrate	Oxaloacetate	Malate	α-Ketoglutarate	Pyruvate	Alanine	Aspartate	Glutamate	Malate/ oxaloacetate
Glucose alone	41 ± 7	49 ± 2	23 ± 4	115 ± 8	110 ± 6	26 ± 3	236 ± 43	3067 ± 169	0.5 ± 0.1
Glucose+albumin	110 ± 11*	8 ± 2*	85 ± 11*	174 ± 24^{+}	188 ± 12*	64 ± 8*	287 ± 25	4128 ± 166*	28 ± 11*

Values represent mean ± S.E.M. in nmol/g. Malate/oxaloacetate is ratio ± S.E.M. *$P < 0.005$, $^{+}P < 0.05$; $n = 8$.

(Table 1). Thus albumin did not stimulate O_2 consumption when NaN_3 was present, indicating that the stimulation of O_2 by albumin is mediated by increased mitochondrial activity.

Another series of experiments was performed in which the effect of polyethylene sulfonate (PES), a water-soluble polyanionic polymer [21], on the stimulation of O_2 consumption by albumin was examined. Polyanionic compounds have been reported to competitively inhibit the interaction of albumin with protein moieties in plasma membranes of cells of the vascular wall [10]. Accordingly, carotid arteries were incubated in the presence or absence of 5 mg/ml PES, with or without albumin (Table 1). PES had no effect on basal O_2 consumption in arteries incubated in normal medium without albumin. After 180 min incubation with albumin and PES, O_2 consumption was increased 36 ± 9% ($n = 3$) above the basal rate; however, this was markedly less than the 115 ± 17% stimulation in the absence of PES. Thus, PES inhibited the stimulatory effect of albumin on O_2 consumption.

3.4. Krebs cycle activity

The stimulation of O_2 consumption by albumin suggested that albumin stimulated mitochondrial oxidative metabolism. To confirm this possibility, the intermediates of the Krebs cycle were measured in the presence and absence of free albumin. The concentrations of key intermediates of the Krebs cycle and associated reactions are given in Table 2. In the presence of albumin, there was a generalized increase (anaplerosis) of the metabolites of the Krebs cycle, as evidenced by significant increases in the concentration of citrate, malate and α-ketoglutarate. The concentration of oxaloacetate, on the other hand, was markedly decreased. However, the ratio of malate/oxaloacetate, which has been reported to reflect the mitochondrial NADH/NAD ratio [22], was markedly increased in arteries incubated with albumin. These observations support the hypothesis that albumin enhanced mitochondrial oxidative metabolism.

Table 2 also indicates that there was a significant increase in the concentration of glutamate, alanine and pyruvate, which are metabolites of subsidiary transaminase reactions of the Krebs cycle. The concentration of aspartate was not increased, consistent with a diminished concentration of oxaloacetate, which is a precursor metabolite to aspartate, and the converse. To exclude the possibility that the difference in amino acid content in the presence and absence of albumin was not due to increased release of intracellular amino acid into the incubation medium, aliquots of incubation medium were assayed for amino acids using the ninhydrin reaction and by enzymatic assay. In four experiments, no free amino acids were detected in the incubation medium at the end of the experimental period either in the presence or in the absence of albumin.

3.5. Effect of albumin on octanoate and acetate oxidation

It was of interest to determine whether albumin affected oxidation of short and medium chain fatty acids. Octanoate is a water-soluble medium chain fatty acid and, therefore, its oxidation by carotid arteries may be determined in the absence of albumin. At a concentration of 0.5 mM octanoate in normal physiological salt solution not containing albumin, the oxidation of octanoate was 1.2 ± 0.04 μmol/g per 180 min ($n = 6$) (or 1.2 ± 0.1 μmol/g per 180 min, 95% confidence interval). When albumin was included in the incubation medium, octanoate oxidation was markedly reduced at 0.29 μmol/g per 180 min. However, since albumin will also bind octanoate its free concentration in the presence of al-

bumin will be less than in its absence and, therefore, the two experimental conditions are not directly comparable. Therefore, octanoate oxidation in the presence of albumin was assessed at a concentration of 1.5 mM octanoate; octanoate oxidation was 0.68 ± 0.10 μmol/g per 180 min ($n = 4$), which was significantly reduced when compared to that at 0.5 mM octanoate in medium not containing albumin ($P < 0.01$). Similarly, at 3.0 and 5.0 mM octanoate in the presence of albumin, octanoate oxidation was 0.26 μmol/g per 180 min and 0.47 μmol/g per 180 min, respectively. (All octanoate oxidation values in the presence of albumin fall below the lower 95% confidence limit for the mean octanoate oxidation value in the absence of albumin.) Thus, across a broad range of concentrations of octanoate, octanoate oxidation was depressed in the presence of albumin. The effect of albumin on acetate oxidation was also investigated because acetate is a water-soluble small chain fatty acid (two carbons) and its oxidation is governed by an enzyme which is different from the enzyme which governs the oxidation of octanoate or palmitate (see Section 4). In the absence of albumin the oxidation of acetate (5 mM) was 2.65 ± 0.25 μmol/g per 180 min ($n = 4$), which was not significantly different than that in the presence of albumin (1.93 ± 0.20 μmol/g per 180 min; $n = 4$, P = NS). Thus, there was a differential effect of albumin on short chain and medium chain fatty acid oxidation.

3.6. Endogenous amino acid

A series of experiments were conducted to examine the effect of albumin on the endogenous glutamate pool. The rationale for these experiments was the observation that the tissue content of glutamate in vascular smooth muscle is substantial (Table 2), and results of a prior study [14] indicating that glutamate may serve as a substrate reserve, and of other studies indicating that the concentration of intracellular amino acids influences the oxidation of glucose [23,24,36]. Since there is significant glucose oxidation in resting muscles, to observe an appreciable change in the intracellular concentration of glutamate experiments were conducted in glucose-free medium. Table 3 gives the content of glutamate in arteries incubated for 180 min in glucose-free and albumin-free medium. There was a significant reduction in glutamate content when compared to arteries incubated in medium containing glucose and albumin. After the 180 min of glucose-free and albumin-free medium, the glutamate-depleted arteries were incubated for an additional 90 min in glucose-free medium but with albumin included. The content of glutamate recovered to near the level before glucose and albumin were deleted. These results and the results given in Table 2 strongly suggest albumin had an effect on the intracellular concentration of glutamate.

3.7. Uptake and degradation of albumin

The time-dependent effect of the albumin on metabolic variables and the effect on intracellular metabolites suggests that albumin exerted an intracellular effect, implying that albumin was taken up by the muscles. This hypothesis was tested by incubating the arteries in medium containing albumin (3%) and 2 μCi of ^{14}C-methylated albumin. At different times, the albumin was washed out of the extracellular space by repeated rinses (60 ml rinses, 2 min between each rinse). Fig. 5 shows the wash-out of counts from the extracellular space; by the fourth rinse, the extracellular space was completely exchanged. The muscles were removed after the eighth rinse and total counts remaining in the muscles determined. At 30 min 9.4 ± 0.5 nmol/g of albumin was measured in the muscles ($n = 4$); by the end of 210 min of incubation 30.9 ± 1.5 nmol/g ($n = 15$) was

Table 3
Effect of albumin on glutamate content

Condition	Glutamate (μmol/g)
Glucose alone (control)	3.07 ± 0.17
Glucose+albumin	4.13 ± 0.17*
Glucose-free alone	$2.39 \pm 0.12^{+}$
Glucose-free+albumin	3.20 ± 0.22

Values represent mean ± S.E.M. In the first two conditions, the arteries were incubated with glucose in the presence or absence of albumin for 180 min. In the second two conditions, the arteries were incubated in glucose-free medium for 180 min after which time albumin was reintroduced into glucose-free medium for an additional 90 min. *Glucose+albumin is significantly greater than all other conditions. $^{+}$Glucose-free alone significantly less than all other conditions. ($n = 8$, $P < 0.05$, ANOVA followed by Newman–Keuls multiple comparisons test).

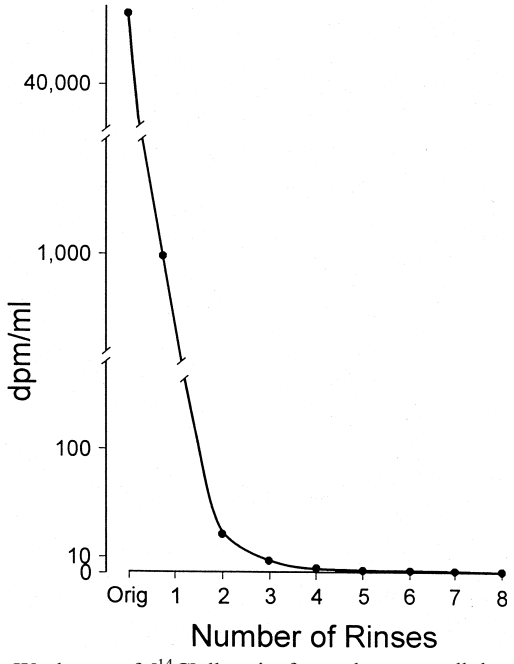

Fig. 5. Wash-out of [^{14}C]albumin from the extracellular space. Representative experiment.

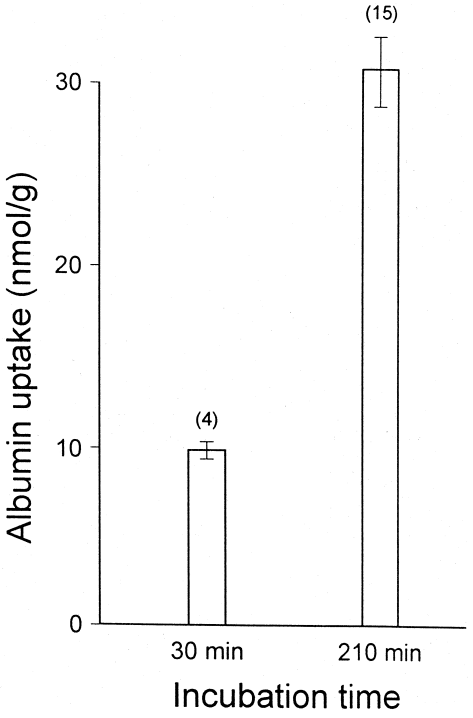

Fig. 6. Uptake of albumin. Bars represent mean ± S.E.M. Numbers in parentheses represent *n*. Value at 210 min significantly greater than 30 min ($P < 0.001$).

measured in the muscles. Fig. 6 shows that the uptake of albumin by the arteries increased with time.

Whether the albumin which was incorporated into the arterial strips underwent proteolytic degradation was assessed by measuring the radioactivity in TCA extracts of arterial strips incubated with albumin and 2 μCi ^{14}C-methylated albumin for 210 min. Substances extracted by TCA would include peptide and amino acid products of albumin degradation. Table 4 indicates that of the 30.9 ± 1.5 nmol/g of albumin incorporated into the tissue 2.0 ± 0.2 nmol/g was TCA-soluble. Samples of incubation medium bathing the arteries were also extracted with TCA; no release of TCA-soluble products into the external medium was detected (data not shown). The energy dependence of albumin degradation was also determined by poisoning the arterial steps with 1 mM iodoacetic acid and 10 mM NaN_3. Such treatment reduces the ATP content of porcine carotid arteries to less than 10% of the control level [25]. As indicated in Table 4, the content of TCA-soluble products in metabolically poisoned arterial strips was markedly reduced when compared to control; and as before, there was no detectable release of TCA-soluble products into the bathing medium (data not shown). These results indicate that there is ATP-dependent proteolytic degradation of albumin. However, total albumin incorporation into the arterial strips was not impaired upon metabolic poisoning (Table 4).

Inasmuch as it was demonstrated that there was some degree of intracellular degradation of albumin it was of interest to ascertain whether the proteolysis of albumin taken up could account for the increased

Table 4
Uptake and degradation of albumin

Condition	*n*	Total uptake (nmol/g)	SDS-soluble incorporation (nmol/g)	TCA-soluble products (nmol/g)
Control	15	30.9 ± 1.5	28.2 ± 1.4	2.04 ± 0.18
NaN_3+IOAA	11	30.6 ± 1.6	30.4 ± 1.6	0.19 ± 0.08*

NaN_3, (sodium azide); IOAA, iodoacetic acid. Values represent mean ± S.E.M. *n* = different arteries from different animals.
*$P < 0.0001$.

content of glutamate and other amino acids in arteries incubated with albumin. This was accomplished by tracking the specific radioactivity of the glutamate pool in arteries incubated in the presence or absence of albumin for 180 min. Since the carbon of the glutamate pool is in equilibrium with the metabolites of the Krebs cycle [17,18] the glutamate pool could be pre-labeled by incubating the arteries with [U-^{14}C]glucose. Dilution of the specific activity of the glutamate pool after incubation with albumin, as compared with that in the absence of albumin, would indicate that unlabeled carbon from albumin had entered the amino acid pool. As shown in Table 5, there was no dilution of the specific activity of the glutamate pool in arteries incubated with albumin, indicating that the increased content of glutamate did not originate from albumin. (The specific activities of alanine and aspartate were also not diluted; data not shown.) Interestingly, a radioactive and ninhydrin-reactive compound with a TLC chromatographic R_f value of 0.70 was detected in perchloric acid extracts from arteries incubated in the absence of albumin, but it was not demonstrable in arteries incubated with albumin. It could account for 407 ± 40 nmol/g ($n = 4$) of the glutamate in the absence of albumin (Table 5).

4. Discussion

Two earlier studies on metabolism of vascular segments have noted metabolic effects of albumin. Hashimoto and Dayton [8], working with rat aorta, first reported the phenomenon of stimulation of O_2 consumption and augmentation of glucose oxidation in aortic segments incubated with albumin. Morrison et al. [26] also reported albumin stimulation of O_2 consumption in rabbit aortae; glucose oxidation, on the other hand, was unaffected. Metabolic effects of albumin on cells incubated in culture have also been reported. In isolated rat hepatocytes, defatted and dialyzed bovine serum albumin (2.5%) increased O_2 consumption [27]. The demonstration in our study that other protein solutions such as porcine skin gelatin and ovalbumin did not exert the effects displayed by mammalian albumin indicates that these phenomena are not attributable to a non-specific effect or to an effect to re-establish normal colloid osmotic pressure. The results with the polyanionic compound polyethylene sulfonate demonstrating blunting of the effect of albumin to stimulate O_2 consumption also exclude these possibilities. Furthermore, the fact that glucose oxidation and O_2 consumption in the absence of albumin remained constant and did not decrease with time argues against the notion that albumin stimulated glucose oxidation by preventing deterioration of the preparation. If this were the case, one would expect the highest metabolic rates to be demonstrable during the earlier periods of incubation, and there should be deterioration of metabolic rates with time in arteries incubated in albumin-free medium. No such deterioration of the preparation was detected. Additionally, we have previously demonstrated that the porcine carotid artery preparation remains stable in albumin-free medium for at least 5 h, without any appreciable decrement in energy metabolism [1,28].

Table 5
Effect of albumin on enrichment of glutamate carbon pool

Condition	Glutamate (nmol/g)	Glutamate specific activity (dpm/nmol)
Albumin absent	3171 ± 43*	53 ± 3
Albumin present	5571 ± 486	60 ± 4

The glutamate pool was labeled by incubating arterial strips in medium containing glucose and 100 μCi/100 ml [U-^{14}C]glucose for 60 min, after which time the medium was washed out and replaced with glucose-free medium in the presence or absence of albumin. The incubation then continued for an additional 180 min. The specific activity of the [U-^{14}C]glucose medium during the labeling period was used to calculate glutamate specific activity. Values represent means ± S.E.M. $n = 4$ different arteries from different animals; *$P < 0.0001$ when compared with albumin present.

4.1. Uptake of albumin

Several pieces of evidence suggest that albumin acted intracellularly. First, the energetic effects of albumin were progressive and increased with time. Second, there was time-dependent uptake of albumin, consistent with time-dependent stimulation of metabolic variables. Third, the stimulation of O_2 consumption by albumin persisted despite wash-out of albumin from the extracellular space. Fourth, there was ATP-dependent breakdown of albumin into TCA-soluble byproducts in the arteries. In cer-

tain other cell types, uptake of albumin has been demonstrated and occurs predominantly by fluid-phase endocytosis [29,30]. Importantly, Schnitzer and Bravos [10] have reported that modified albumin can indeed bind to glycoprotein receptors on the plasma membrane of cultured rat aortic smooth muscle cells and that the albumin can be internalized into the cytosol and degraded intracellularly. Our results are consistent with this study. The fact that there was not an immediate and maximal step-up in metabolic rates of the arteries on contact with albumin suggests that several antecedent biochemical events were required before the metabolic effects were manifested. It was demonstrated that the albumin taken up by the arteries undergoes some degree of ATP-dependent proteolysis. ATP-dependent breakdown of proteins taken up into proteasomes of cells is a well-described phenomenon [31]. However, the findings of previous studies suggest that intact albumin may be producing the metabolic effects observed in the present investigation by interacting either with fatty acid components or with enzyme and other protein components of the mitochondrial membrane [32–34]. Whether intact albumin or its breakdown products mediate the metabolic effects remains to be determined.

The uptake of albumin into the arteries was not found to be ATP-dependent. Rather, it is likely that albumin was taken up into the cells by endocytotic nucleation of the cell membranes. Spontaneous vesicle formation and internalization of vesicles into the cytosol of cells is determined by the thermodynamics of fluid-phase interactions and the viscosity of the extracellular fluids relative to that of the cytosol [35].

4.2. Fatty acid metabolism

Albumin had pronounced effects on several aspects of the metabolism of the arteries. Albumin stimulated O_2 consumption and glucose oxidation, altered the disposition of intracellular amino acids and inhibited oxidation of the medium chain fatty acid octanoate. It is possible that albumin also had an inhibiting effect on the oxidation of palmitate, accounting for its low rate of oxidation by the arteries and the lack of augmentation of its oxidation upon contractile activation of the muscle. The rate of oxidation of exogenous palmitate in resting artery was $\sim$0.17 nmol/min/g while the maximum rate of O_2 consumption was $\sim$0.61 μmol/min/g. If it is assumed that 23 mol of O_2 are required to oxidize 1 mol of palmitate, then the oxidation of palmitate accounted for only a trivial percentage ($< 3\%$) of the O_2 consumption in resting muscle. By contrast, in the absence of albumin, fatty acid oxidation accounts for the majority of the O_2 consumption and fatty acid oxidation increases during contraction [1,2]. The demonstration that the arterial strips contained a substantial quantity of free palmitate and that palmitate incorporated into all the cellular lipid classes indicates that uptake of palmitate across the sarcolemma was not a limiting factor of palmitate oxidation. The uptake of palmitate far exceeded its rate of oxidation. That palmitate was a relatively ineffective oxidative substrate under these experimental conditions was also indicated by the observation that its presence or absence did not affect the oxidation of glucose. Under most conditions, exogenous fatty acid oxidation typically results in suppression of glucose oxidation in other tissues [35,36], and vascular smooth muscle when albumin is absent [11].

It is possible to explain the suppression of exogenous palmitate and octanoate oxidation on the basis of known regulatory mechanisms of fatty acid metabolism [37]. Transport of these fatty acids into the mitochondrial matrix is rate-limiting for their oxidation and is mediated by carnitine palmitoyl transferase (EC 2.3.1.21). This enzyme, in turn, is negatively regulated by the concentration of malonyl CoA. The level of malonyl CoA is determined by the activity of acetyl CoA carboxylase, which catalyzes the carboxylation of acetyl CoA to form malonyl CoA. A positive effector of acetyl CoA carboxylase is citric acid. When levels of citric acid and acetyl CoA are elevated the activity of acetyl CoA carboxylase is enhanced leading to an increased level of malonyl CoA, which will then feedback and inhibit carnitine palmitoyl transferase, limiting further metabolism and oxidation of fatty acid within the mitochondria. Thus, when there is anaplerosis of the Krebs cycle and an increase in citric acid and acetyl CoA, as occurs in porcine carotid artery incubated with albumin, medium chain and long chain fatty acid oxidation may decrease. On the other hand, short chain fatty acid (acetate) oxidation was not inhibited by albumin.

Transport of acetate into the mitochondrial matrix is governed by carnitine acetyl transferase (EC 2.3.1.7). The regulation of this enzyme is different from that of carnitine palmitoyl transferase, and malonyl CoA has not been reported to exert an inhibitory effect [38]. So it is likely that its activity proceeded unimpeded in the presence of albumin.

4.3. Amino acid metabolism

Albumin treatment resulted in increases in the content of glutamate and alanine. The evidence suggested that albumin may not have changed the total amino acid content per se, but rather, it altered the distribution of amino acid nitrogen within the endogenous amino acid pool. Glutamate is the major substrate for transaminase and other reactions and its nitrogen and carbon may be dispersed to other cellular amino acids. Albumin could have affected the interconversions between intracellular amino acids. Further investigation is required to define the mechanism by which this occurred.

4.4. Glucose oxidation

The altered content and disposition of amino acids induced by albumin may be the link to the augmentation of glucose oxidation. Previous studies [23] have indicated that the rate of oxidation of glucose by porcine carotid artery is affected by the malate–aspartate shuttle. In turn, the activity of the malate–aspartate shuttle is affected by the concentration of intracellular glutamate and aspartate [23,24,36], which are primary reactants of the shuttle. The reduction in the production of lactate as glucose oxidation was increasing is consistent with increased activity of the malate–aspartate shuttle since the function of the shuttle is to transport reducing equivalents from the cytosol into the mitochondria [23,24]. This would result in reduced formation of lactate and an increased intracellular concentration of pyruvate, as we have shown.

In summary, novel and important effects of serum albumin on substrate utilization and the metabolic economy of vascular smooth muscle have been described. Evidence has been presented indicating that albumin is taken up by the muscles to exert intracellular effects. The mechanisms by which albumin exerts these actions remain to be elucidated. Nevertheless, the results may have important implications with regard to vascular disease states, such as hypertension, diabetes and atherosclerosis, in which possible alterations in handling or processing of serum albumin may result in a switch in the pattern of substrate utilization and oxidation, possibly contributing to the pathological process.

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