substrates for transglutaminase. *J Protein Chem* **6**: 353–359, 1987.
10. Miele L, Cordella-Miele E and Mukherjee AB, Uteroglobin: structure, molecular biology, and new perspectives on its function as a phospholipase A2 inhibitor. *Endocrine Rev* **8**: 474–490, 1987.
11. Miele L, Cordella-Miele E, Facchiano A, and Mukherjee AB, Novel anti-inflammatory peptides from the region of highest similarity between uteroglobin and lipocortin I. *Nature* **335**: 726–730, 1988.
12. Metafora S, Peluso G, Persico P, Ravagnan G, Esposito C and Porta R, Immunosuppressive and anti-inflammatory properties of a major protein secreted from the epithelium of the rat seminal vesicles. *Biochem Pharmacol* **38**: 121–131, 1989.
13. Metafora S, Porta R, Ravagnan G, Peluso G, Tufano MA, De Martino L, Ianniello R and Galdiero F, Inhibitory effect of SV-IV, a major protein secreted from the rat seminal vesicle epithelium on phagocytosis and chemotaxis of human polymorphonuclear leukocytes. *J Leuk Biol* **46**: 409–416, 1989.
14. Galdiero F, Tufano MA, De Martino L, Capasso C, Porta R, Ravagnan G, Peluso G and Metafora S, Inhibition of macrophage phagocytic activity by SV-IV, a major protein secreted from the rat seminal vesicle epithelium. *J Reprod Immunol* **16**: 269–284, 1989.
15. Levin SW, Butler JD, Schumacher UK, Wightman PD and Mukherjee AB, Uteroglobin inhibits phospholipase A2 activity. *Life Sci* **38**: 1813–1819, 1986.
16. Manjunath R, Levin SW, Kumaroo KK, Butler JD, Donlon JA, McDonald M, Fujita R, Schumacher UK and Mukherjee AB, Inhibition of thrombin-induced platelet aggregation by uteroglobin. *Biochem Pharmacol* **36**: 741–746, 1987.
17. Pinon JF, *In vivo* study of platelet aggregation in rats. *J Pharmacol Meth* **12**: 79–84, 1984.
18. Rotman A, Meyer FA, Gitler G and Silberburg A, *Platelets. Cellular Response Mechanisms and Their Biological Significance*. Wiley, New York, 1980.

Biochemical Pharmacology, Vol. 40, No. 5, pp. 1161–1164, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Thyroid hormone analogue SKF L-94901: effects on amino acid and carbohydrate metabolism in rat skeletal muscle *in vitro*

(*Received* 8 *December* 1989; *accepted* 10 *May* 1990)

An analogue of T_3 [SKF L-94901 (SKF 901)] has recently been developed [1]. SKF 901 induces substantial increases in the activity of the hepatic, but not cardiac, mitochondrial enzyme cytochrome *c* 3-phosphoglycerate oxido-reductase and decreases plasma levels of both cholesterol and thyroid stimulating hormone in hypothyroid rats [1]. SKF 901 has equal affinities for receptors in heart and liver. However, SKF 901 stimulates liver, but has little effect on cardiac, function [1].

Carbohydrate, protein and amino acid metabolism is greatly influenced by thyroid hormones *in vivo*. For example, hyperthyroidism causes impaired glucose tolerance in both man and experimental animals [2–7]. Recently, it has been reported that the sensitivity of glycogen synthesis to insulin is decreased in incubated soleus muscle preparations isolated from hyperthyroid rats [8]. Furthermore, hyperthyroidism increases the responsiveness of both the rates of glycolysis and glucose transport to insulin [8]. It is not known how T_3 affects these processes. T_3 increases the rate of protein turnover either by increasing the rate of protein degradation [9, 10] or decreasing the rate of protein synthesis [11] and this largely occurs in skeletal muscle. Therefore, it is important to identify the mechanisms employed by T_3 to alter metabolic regulatory processes in skeletal muscle.

If SKF 901 has T_3-like effects in skeletal muscle then this agent may be utilised to increase the understanding of T_3 action in this tissue. No study has investigated the effects of SKF 901 on carbohydrate and amino acid metabolism in skeletal muscle. Therefore, such a study was undertaken. Rats were administered with either T_3 or SKF 901 for 5 days and the efficacy of both agents *in vivo* was similar (based on relative potency values for T_3 and SKF 901 [1]). The effects of both agents on the rates of lactate formation, glycogen synthesis and glutamine release were studied in stripped soleus muscle *in vitro*. Plasma levels of glucose and lactate and the content of glutamine and glycogen in gastrocnemius and soleus muscles, respectively, were also measured.

Materials and Methods

Rats were made hyperthyroid by daily intraperitoneal injection of T_3 (0.65 μg/g body wt) [8]. SKF 901 (3.25 μg/g body wt) was initially dissolved in small volume of alkaline H_2O before dilution to the appropriate concentration with sterile 0.9% (w/v) NaCl before intraperitoneal administration of SKF 901 at a similar efficacy as compared with T_3 (this calculation was based on the relative potency of the two agents [1]). The time between the last injection and preparation of strips of soleus muscle was 16 hr. Soleus muscle strips were prepared from 14 hr fasted male Wistar rats (Harlan-Olac, Bicester, U.K.; 160–180 g) as previously described [12–14]. The tendons of the muscles were ligated before attachment to stainless steel clips. Muscle strips were pre-incubated in Erlenmeyer flasks containing 3.5 mL Krebs–Ringer bicarbonate buffer plus (mM) *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (7), pH 7.4, glucose (5.5), pyruvate (5), 1.5% (w/v) de-fatted bovine serum albumin. In one experiment (effects of T_3 on rates of glutamine release from soleus muscle) pyruvate was omitted from the pre-incubation buffer and this resulted in lower rates of glutamine release. Other unpublished experiments (Parry-Billings *et al.*) have established that the omission of pyruvate from the pre-incubation medium dramatically reduces the variability of rates of glutamine release in control incubated isolated stripped soleus muscles but does not alter the magnitude of the effects of agents that alter rates of glutamine release. Flasks were sealed and aerated continuously with O_2:CO_2 (19:1, v/v). After pre-incubation of muscles in this medium for 30 min, at 37° in an oscillating water bath, the muscle strips were transferred to similar vials containing identical medium (except pyruvate was omitted) with added [U-^{14}C]glucose (0.5 μCi/mL) and various concentrations of insulin (see

Results). The flasks were sealed, re-gassed for the initial 15-min period and incubated for 1 hr. The details of the incubation procedures have previously been described in detail [13, 14]. At the end of the incubation period, muscles were blotted and rapidly frozen in liquid nitrogen. The concentration of lactate in the incubation medium and plasma was determined spectrophotometrically [15] and [U-^{14}C]glucose incorporated into glycogen (glycogen synthesis) was measured [16]. Glycogen content was determined as previously described [17]. Plasma glucose and glutamine content in plasma, incubation medium and gastrocnemius muscle were measured as previously described [18]. The gastrocnemius muscle was sampled immediately after the rats were killed. This muscle was taken because it can be rapidly dissected and so a measure of the effect of both agents on *in vivo* muscle glutamine metabolism could be monitored. However, it should be noted that the soleus muscle is largely populated with slow contracting type I fibres but the gastrocnemius contains both type I and type II fibres.

Results are given as mean ± SE. The significance of differences between means were analysed by the non-paired Student's *t*-test.

Results and Discussion

The effect of insulin on the rates of lactate release and glycogen synthesis in the isolated soleus muscle, from control and treated animals, are given in Table 1. In control muscles insulin (10,000 μUnits/mL) maximally stimulated the rates of glycolysis and glycogen synthesis by 43% and 447%, respectively (Table 1). For muscles removed from animals treated with T_3 for 5 days, the rates of lactate release at all insulin concentrations were markedly increased (2-fold). In addition, the responsiveness of glycolysis to insulin was increased about 200%. After 5 days administration of SKF 901, the responsiveness of glycolysis was increased about 40%. Although SKF 901 significantly increased the rates of glycolysis at 10, 1000 and 10,000 μUnits of insulin/mL, the magnitude of these increases were about 5-fold less than those caused by T_3 administration.

Administration of T_3 caused a significant decrease in the rates of glycogen synthesis at all concentrations of insulin. In contrast, SKF 901 treatment only caused a decrease in the rate of glycogen synthesis at a sub-maximal level of insulin (100 μUnits/mL). The concentrations of insulin required to give the same response as that observed at 100 μUnits of insulin/mL under the control conditions were approximately 490 μUnits/mL and 2100 μUnits/mL for muscles from SKF 901 and T_3-treated muscles respectively. This indicates a decrease in the sensitivity of glycogen synthesis to insulin which was particularly marked in the T_3 treated animals. This is consistent with the finding that the glycogen contents of soleus muscle are significantly decreased by both treatments (see Table 2).

T_3 administration for 5 days increased the plasma levels of both glucose and lactate (Table 2). However, SKF 901 elevated only the plasma levels of lactate. The rate of glutamine release from the incubated soleus muscle was increased by T_3, but unaffected by SKF 901 treatment. Skeletal muscle and plasma glutamine content were unchanged by both treatments.

The results of Underwood and co-workers [1] demonstrated that the T_3 analogue (SKF 901) had much less effect on metabolism in the heart than the liver. However, the present study demonstrated that administration of SKF 901 *in vivo* alters different aspects of insulin-stimulated carbohydrate metabolism in skeletal muscle *in vitro*. Both T_3 and SKF 901 increased the rates of glucose conversion to lactate (glycolysis; see Table 1). However, SKF 901 is much less effective than T_3. If these effects can be extrapolated to the *in vivo* situation, the effect of increased rates of lactate formation by skeletal muscle would be to stimulate the rate of gluconeogenesis and hence Cori cycle activity [19]. Cori cycle activity results in energy expenditure since more ATP is hydrolysed in conversion of glucose to lactate (in muscle) than is produced in conversion of lactate to glucose (in liver) [20]. Of interest, T_3 administration increased the rate of glucose conversion to lactate, at all concentrations of insulin, in the isolated soleus muscle (about 200%) whereas SKF 901 only increased the rates about 40%: such a difference in effectiveness is observed for stimulation of oxygen consumption by these two agents in the intact animal [1]. It is tempting to speculate that an increase in the Cori cycle activity may play an important role in increasing energy expenditure caused by T_3 and SKF 901 and that this could explain, at least in part, the increase in the rates of glycolysis in muscle.

Glutamine is the most abundant amino acid in the body. It has the highest concentration in the plasma, and accounts

Table 1. Effects of Tri-iodothyronine (T_3) and SKF 901 administration to rats on the rates of lactate formation and glycogen synthesis in stripped soleus muscle in the presence of various concentrations of insulin

Insulin concentration (μUnits/mL)	Rates of glycolysis (μmol/hr/g wet wt)		
	Control	SKF 901	T_3
10	8.81 ± 0.47	10.24 ± 0.46*	18.30 ± 1.15†
100	12.71 ± 0.49	13.29 ± 0.66	25.97 ± 1.63†
1000	12.46 ± 0.70	14.79 ± 0.71*	26.01 ± 1.77†
10,000	12.63 ± 0.50	15.48 ± 0.35*	25.68 ± 0.25†
	Rates of glycogen synthesis (μmol/hr/g wet wt)		
10	1.17 ± 0.08	1.23 ± 0.14	0.81 ± 0.07*
100	3.95 ± 0.43	2.46 ± 0.14*	2.65 ± 0.25*
1000	5.21 ± 0.25	5.06 ± 0.41	3.76 ± 0.22†
10,000	6.41 ± 0.29	5.58 ± 0.47	4.44 ± 0.24†

Rats were treated with either drug for 5 days.
Values are presented as means ± SE for five separate incubations.
The statistical significance of the difference from control values is denoted by * $P < 0.05$ and † $P < 0.001$.

Table 2. Effects of tri-iodothyronine (T_3) and SKF 901 administration for 5 days on the plasma concentrations of glucose, glutamine and lactate, rates of glutamine release from incubated soleus muscle and glutamine and glycogen content in muscle

Measurement	Control	SKF 901	T_3
Plasma glucose (mM)	4.71 ± 0.14 (16)	4.16 ± 0.21 (5)	5.61 ± 0.29 (11)*
Plasma glutamine (mM)	1.06 ± 0.05 (8)	0.92 ± 0.06 (8)	0.85 ± 0.03 (9)*
Plasma lactate (mM)	1.09 ± 0.08 (9)	1.75 ± 0.22 (5)*	1.94 ± 0.24 (11)*
Soleus glycogen content (μmol/g)	26.5 ± 0.6 (4)	22.8 ± 1.0 (5)	23.2 ± 1.2 (5)*
Soleus glutamine release (μmol/min/g)	53.3 ± 4.6 (17) 28.1 ± 1.8 (32)	52.1 ± 5.7 (16)	47.1 ± 3.0 (16)*
Gastroc. glutamine content (μmol/g)	4.09 ± 0.2 (8) 3.37 ± 0.8 (14)	3.88 ± 0.2 (8)	3.03 ± 0.1 (11)

for more than 50% of the intracellular free amino acid content in skeletal muscle. It is suggested that the nitrogen and some of the carbon from branch chain amino acids (rather than degradation of protein) is used to synthesise glutamine in muscle. T_3 administration was without effect on gastrocnemius glutamine content, but significantly increased the rates of glutamine release from the stripped soleus muscle *in vitro* (Table 2). Hyperthyroidism in man greatly alters whole body protein metabolism and significantly increases glutamine efflux from leg muscle [11]. T_3, but not SKF 901, affected glutamine metabolism in skeletal muscle whereas both agents modulated glucose metabolism, therefore, it might be speculated that T_3 employs different mechanisms to regulate carbohydrate and amino acid metabolism in muscle. Since SKF 901 affects carbohydrate metabolism, but not amino acid metabolism, then this thyroid hormone analogue may be of value as a novel experimental agent to elucidate the mechanisms by which T_3 regulates the activities of the glucose transporter, key enzymes of glycolysis and glycogen synthase in skeletal muscle.

In summary, hyperthyroidism increased the rate of glycolysis and decreased glycogen synthesis in isolated incubated rat soleus muscle preparations. SKF 901 also increased glycolysis, but the stimulation was 5-fold less than in T_3-treated muscles. Hyperthyroidism increased the rate of glutamine release from skeletal muscle, but SKF 901 did not affect glutamine metabolism.

Acknowledgements—We thank Dr Anthony Underwood, Smith Kline French Ltd for providing the thyroid hormone analogue (SKF-94901) and for helpful discussions. We thank Professor E. M. Southern for provision of facilities.

Department of Biochemistry
University of Oxford
South Parks Road
Oxford OX1 3QU
U.K.

BRENDAN LEIGHTON*
GEORGE D. DIMITRIADIS
MARK PARRY-BILLINGS
JANE BOND
PAUL KEMP
ERIC A. NEWSHOLME

REFERENCES

1. Underwood AH, Emmett JC, Ellis D, Flynn DB, Leeson PD, Benson GM, Novelli R, Pearce NJ and Shah VP, A thyromimetic that decreases plasma cholesterol levels without increasing cardiac activity. *Nature* **324**: 425–429, 1986.
2. Osei K, Falko J, O'Drisio T and Adam D, Decreased serum C-peptide/insulin molar ratios after oral glucose ingestion in hyperthyroid patients. *Diabetes Care* 7: 471–475, 1984.
3. Dimitriadis GD, Baker B, Marsh H, Mandarino L, Rizza R, Bergman R, Haymond M and Gerich J, Effect of thyroid hormone excess on action, secretion, and metabolism of insulin in humans. *Am J Physiol* **248**: E593–E601, 1985.
4. Shen D and Davidson M, Hyperthyroid Grave's disease causes insulin antagonism. *J Clin Endocrinol Metab* **60**: 1038–1041, 1985.
5. Muller M and Seitz H, *In vivo* glucose turnover in hypo- and hyperthyroid starved rats. *Pfluegers Arch* **386**: 47–52, 1980.
6. Muller M, Paschen U and Seitz H, Thyroid hormone regulation of glucose homeostasis in the miniature pig. *Endocrinology* **112**: 2025–2113, 1983.
7. Okajima F and Ui M, Metabolism of glucose in hyper- and hypothyroid rats *in vivo*: glucose turnover values and futile cycle activities with ^{14}C and ^{3}H-labelled glucose. *Biochem J* **182**: 565–576, 1979.
8. Dimitriadis GD, Leighton B, Vlachonikolis IG, Parry-Billings M, Challiss RAJ, West D and Newsholme EA, Effects of hyperthyroidism on the sensitivity of glycolysis and glycogen synthesis in the soleus muscle of the rat. *Biochem J* **253**: 87–92, 1988.
9. Carter WJ, Van der Weijden BWS and Faas FH, Effect of experimental hyperthyroidism on protein turnover in skeletal and cardiac muscle. *Metabolism* **29**: 910–915, 1980.
10. Goldberg AI, Tischler ME, DeMartino G and Griffen G, Hormonal regulation of protein degradation and synthesis in skeletal muscle. *Fed Proc* **39**: 31–36, 1980.
11. Morrison WL, Gibson JNA, Jung RT and Rennie MJ, Skeletal muscle and whole body protein turnover in thyroid disease. *Eur J Clin Invest* **18**: 62–68, 1985.
12. Crettaz M, Prentki M, Zaninetti D and Jeanrenaud B, Insulin resistance in soleus muscle from obese Zucker rats. *Biochem J* **186**: 525–534, 1980.
13. Espinal J, Dohm L and Newsholme EA, Sensitivity to insulin of glycolysis and glycogen synthesis of isolated soleus muscle strips from sedentary, exercise and exercised-trained rats. *Biochem J* **121**: 453–458, 1983.
14. Challiss RAJ, Espinal J and Newsholme EA, Insulin sensitivity of rates of glycolysis and glycogen synthesis in soleus, epitrochlearis and hemi-diaphragm muscles of the rat. *Biosci Reports* **3**: 675–679, 1983.
15. Engel P and Jones J, Causes and elimination of erratic blanks in enzymatic metabolite assays involving the use of NAD^+ in alkaline hydrazine buffers. *Anal Biochem* **88**: 475–484, 1978.
16. Ceudet G, Loten E, Jeanrenaud B and Renold A,

* Author for correspondence.

Decreased basal and non-insulin stimulated glucose uptake and metabolism by skeletal soleus muscle isolated from obese-hyperglycaemic mice. *J Clin Invest* **58**: 1078–1088, 1976.
17. Leighton B, Kowalchuk JM, Challiss RAJ and Newsholme EA, Circadian rhythm in sensitivity of glucose metabolism to insulin in rat soleus muscle. *Am J Physiol* **255**: E41–E45, 1988.
18. Parry-Billings M, Leighton B, Dimitriadis GD, DeVasconcelos PRL and Newsholme EA, Skeletal muscle glutamine metabolism during sepsis in the rat. *Int J Biochem* **21**: 419–423, 1989.
19. Huang M and Lardy H, Effect of thyroid status on the Cori-cycle, glucose-alanine cycle, and futile cycle of glucose metabolism in rats. *Arch Biochem Biophys* **209**: 41–51, 1981.
20. Newsholme EA and Leech AR, In: *Biochemistry for the Medical Sciences*, p. 205. Wiley, Chichester, U.K., 1983.

Biochemical Pharmacology, Vol. 40, No. 5, pp. 1164–1167, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Doxorubicin-3′-NH-oestrone-17-oxime-ethyl-carbonyl, a doxorubicin-oestrone conjugate that does not redox cycle in rat liver microsomes

(*Received* 15 *March* 1990; *accepted* 15 *May* 1990)

Doxorubicin (Adriamycin®) is a broad spectrum antitumour agent commonly used in the treatment of advanced cancers, either as a single agent or in combination chemotherapy. Unfortunately, a dose-limiting factor in the clinical use of doxorubicin is cardiotoxicity [1], an event that is suggested to be associated with doxorubicin free radical formation [2]. Various approaches have been adopted to decrease this cardiotoxicity through the use of vehicles and formulations predicted to minimize drug uptake and/or damage to heart tissue. These include the use of doxorubicin-entrapped cardiolipin liposomes [3], doxorubicin-*N*-(hydroxypropyl)methylacrylamide copolymers [4] and doxorubicin–iron chelates [5]. An alternative strategy for minimizing doxorubicin-mediated cardiotoxicity is to target this agent to specific tumours. Doxorubicin is one of the preferred treatments for progressive metastatic breast cancer, a tumour type that is sensitive to oestrogen therapy [6]. With this in mind, we have developed a doxorubicin-3′-NH-oestrone-17-oxime-ethyl-carbonyl (Dox-Oes; Fig. 1a) covalent adduct with the aim of targeting doxorubicin (Fig. 1b) to oestrogen receptor positive breast cancer cells. We report here the lack of free radical generation and redox cycling by this conjugate.

Materials and Methods

Drugs and chemicals. Dox-Oes was prepared by reacting the 3′-amino group of doxorubicin with the carboxyl group of 17-carboxyalkyloxime-oestrone [7]. Biochemicals and reagents were purchased from either the Sigma Chemical Co. (Poole, Dorset, U.K.) or Boehringer Mannheim (Lewes, Sussex, U.K.). Bio-Rad protein assay reagent was purchased from Bio-Rad laboratories (D-8000 München, F.R.G.).

Electron spin resonance (esr) studies. ESR studies were carried out using a Varian E109 X-band spectrophotometer at a microwave frequency of 9.5 GHz at ambient temperature. The 1-mL anaerobic incubation mixtures consisted of 250 mM drug, 4 mM NADPH and rat liver microsomal protein (typically 6 mg) prepared as described by [8] in 200 mM phosphate buffer (pH 7.4) containing 5% Tween 20.

NADPH utilization. This was done as described previously [9]. The reaction mixture at 37° consisted of 50 μM drug, 0.1 mM NADPH, 7.5 mM nicotinamide (to inhibit NADPH degradation), 90 μg microsomal protein and buffer (100 mM sodium phosphate/225 mM KCl, 5% Tween 20, pH 7.4). Basal rate NADPH oxidation was determined under identical conditions but without drug.

Superoxide anion formation. Superoxide anion formation was measured at 37° by two methods as previously described [9]. (i) Reduction of acetylated cytochrome *c*. The 1-mL incubation mixture consisted of 1 mM NADPH, 0.07 mM acetylated cytochrome *c*, 19 μg microsomal protein and 50 μM drug in buffer (50 mM Tris/150 mM KCl, 5% Tween 20, pH 7.4). (ii) Oxidation of adrenaline to adrenochrome. The 1-mL incubation mixture consisted of 1 mM NADPH, 2 mM adrenaline bitartrate, 70 μg microsomal protein and 50 μM drug all in buffer (50 mM Tris/150 mM KCl, 1 mM EDTA, 5% Tween 20, pH 7.4). Basal rate superoxide anion formation in liver microsomes was determined as described above without addition of drugs.

Results and Discussion

Table 1 shows that doxorubicin stimulated basal rate NADPH oxidation 3-fold and superoxide anion generation 2-fold in rat liver microsomes. This tissue fraction is a rich source of cytochrome P-450 reductase, an enzyme known to participate in the activation of doxorubicin. The results are consistent with a doxorubicin redox cycle that results in reactive oxygen generation at the expense of cellular reducing equivalents as has been previously described [2, 10]. Detection of metabolically generated doxorubicin free radicals in the absence of oxygen (Fig. 2a) further supports this since under these conditions NADPH reduces doxorubicin to a semiquinone (free radical) which is detectable by esr. The asymmetry of the doxorubicin spectrum is consistent with previous results [11] and is suggested to be a consequence of immobilization of the doxorubicin semiquinone generated in a microsomal environment. In contrast, Table 1 shows that Dox-Oes does not redox cycle, as indicated by no stimulation of basal rate NADPH oxidation or superoxide anion generation in the presence of this conjugate. In support of this, Dox-Oes does not generate an esr-detectable free radical intermediate (Fig. 2b). Furthermore, Dox-Oes actually inhibits doxorubicin free radical formation (Fig. 2c). The concentrations of doxorubicin, Dox-Oes and microsomal protein used for the esr studies was considerably greater than that used for measuring the redox activity of these compounds (see Materials and Methods). This was to ensure that a doxorub-