The cyclo-oxygenase inhibitors indomethacin and ibuprofen inhibit the insulin-induced stimulation of ribosomal RNA synthesis in L6 myoblasts

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Insulin stimulated total RNA accretion and the incorporation of [³H]uridine into RNA in L6 skeletal-muscle myoblasts. Incorporation of uridine into the rRNA was measured after either separation of 18 S and 28 S rRNA species by agarose-gel electrophoresis or separation of dissociated 40 S and 60 S ribosomal subunits on sucrose density gradients. Both methods showed a stimulation by insulin of uridine incorporation into the RNA of the two subunits. Two non-steroidal anti-inflammatory drugs, indomethacin and ibuprofen, which inhibit the metabolism of arachidonic acid by the cyclo-oxygenase pathway, inhibited the insulin-induced accretion of total cellular RNA and the incorporation of uridine into the RNA of both ribosomal subunits. The effect of insulin was observed both by using a tracer dose of [³H]uridine (5 μ M) and in the presence of a high concentration (1 mM) of uridine to minimize possible changes in intracellular precursor pools. Neither insulin nor indomethacin was found to affect the incorporation of uridine into the total intracellular nucleotide pool, or the conversion of uridine into UTP. The ability of inhibitors of arachidonic acid metabolism to prevent insulin-induced increases in RNA metabolism suggests that a prostaglandin or other eicosanoid is involved in the signal mechanism whereby insulin stimulates RNA synthesis.

INTRODUCTION

The stimulation of muscle protein synthesis is central to the effects of insulin on growth (Manchester, 1970; Ashford & Pain, 1986). This effect of the hormone occurs both in the whole animal (Garlick *et al.*, 1983) and in isolated muscles (Reeds & Palmer, 1983) as well as in muscle cells in culture (Palmer & Bain, 1989). The effect of insulin *in vivo* has been shown to involve both a rapid stimulation of the initiation of translation (Jefferson, 1980; Harmon *et al.*, 1984) and an increase in ribosome synthesis related to the longer-term control of protein accretion (Ashford & Pain, 1986). Insulin thus has profound effects on both short- and long-term control of protein synthesis. Similarly, in fibroblasts in cell culture, insulin has been found to have rapid effects on translation by existing ribosomes and slower effects on protein synthesis, which are dependent on RNA synthesis (DePhilip *et al.*, 1979; Hesketh *et al.*, 1986).

Little, however, is understood of the series of membrane and cytoplasmic events which comprise the signal mechanisms by which insulin brings about these effects. In cultured fibroblasts there is evidence for an involvement of diacylglycerol and protein kinase C with the rapid activation of existing ribosomes (Hesketh *et al.*, 1986, 1988), whereas the effects of insulin on muscle protein synthesis *in vitro* are blocked by the non-steroidal anti-inflammatory drug indomethacin, which inhibits eicosanoid formation through metabolism of arachidonic acid by the cyclo-oxygenase pathway (Reeds & Palmer, 1983). Similarly, in post-absorptive rats infusion of insulin for only 30 min increased rates of protein synthesis, and this response too was blocked by indomethacin (Reeds *et al.*, 1985). These studies

implicated a cyclo-oxygenase product of arachidonic acid (prostaglandin F_{2 α}) in the control of protein synthesis by insulin, and because of the short duration of the experiments the effects were ascribed to changes in the activity of the existing population of ribosomes rather than to an effect on ribosome synthesis. The involvement of cyclo-oxygenase products in the synthesis and accretion of RNA was suggested by the observation that the total RNA content of both normal and hypertrophying muscles of rats was decreased in response to another cyclo-oxygenase inhibitor, fenbufen (McMillan *et al.*, 1988) and by the ability of indomethacin to inhibit insulin-induced increases in total RNA synthesis and accretion in the L6 line of skeletal-muscle myoblasts (Palmer & Bain, 1989). No attempt was made in either of these studies to isolate the rRNA; thus the possibility that the effects were confined to non-ribosomal RNA species could not be precluded. The object of the present study was to examine the effects of two cyclo-oxygenase inhibitors, indomethacin and ibuprofen, on the incorporation of uridine into rRNA.

EXPERIMENTAL

Materials

L-[5-³H]Uridine was purchased from Amersham International (Amersham, Bucks., U.K.). All tissue-culture materials were purchased from Gibco (Paisley, Scotland, U.K.). Insulin (human Actrapid) was obtained from Farillon Ltd. (Romford, Essex, U.K.). Ibuprofen was purchased from a local pharmacy; indomethacin, uridine and fetuin were from Sigma Chemical Co. (Poole, Dorset, U.K.).

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Cells and labelling conditions

L6 cells (European Collection of Animal Cell Cultures, Porton, Wilts., U.K.) were treated on arrival as described previously (Palmer & Bain, 1989) and frozen in liquid N₂ until required. The following experimental protocol was used throughout.

Cells were grown in Dulbecco's modified Eagle's medium containing 12% (v/v) foetal-calf serum, and experiments were performed on cells 7–8 days after thawing. All experiments were performed 48–72 h after subculture, while the cells were sub-confluent. At this time the medium was removed, the cell monolayer was washed once with serum-free medium, and serum-free Dulbecco's medium containing fetuin (1 mg/ml) (Florini & Roberts, 1979) was added. Then 18 h later the following additions were made to the appropriate cultures: insulin [50 m-units/ml in phosphate-buffered saline (154 mM-NaCl, 2 mM-NaH₂PO₄, 8 mM-Na₂HPO₄, pH 7.4)] to give a final concentration of 100 μ -units/ml; indomethacin (30 μ mol/ml in ethanol, final concn. 50 μ M); ibuprofen (30 μ mol/ml in phosphate-buffered saline, final concn. 100 μ M) and [³H]uridine (final concn. either 5 μ M + 0.2 μ Ci/ml of medium, or 1 mM + 1 μ Ci/ml of medium). Subsequently incubations continued for up to 6 h.

For experiments to measure the incorporation of uridine into the intracellular pools and into the total cellular RNA, cells were plated at $(7-8) \times 10^4$ cells/ml on to 50 mm-diam. Petri dishes (2.5 ml of medium/dish). Incubation times varied in these experiments as detailed in the appropriate Tables. For experiments involving the separation of 18S and 28S rRNA or the separation of 40S and 60S ribosomal subunits, cells were plated at the same density but in 8 ml of medium on to 90 mm-diam. dishes. The incubation time in all these experiments was 6 h.

RNA analysis

(a) **Labelling of UTP and total RNA analysis.** Total RNA-associated and free intracellular uridine were separated on the basis of solubility in HClO₄ as described previously (Palmer & Bain, 1989). RNA content and associated radioactivity were determined respectively by measuring the absorbance at 260 and 232 nm (Munro & Fleck, 1969) and by scintillation counting.

UTP was isolated by h.p.l.c. from 0.2 M-HClO₄-soluble cell extracts containing free nucleotides. Samples (200 μ l) were applied to a 250 mm $\times$ 4.6 mm column of Partisil SAX 10 μ (de Corte *et al.*, 1985), and 1.7 ml (1 min) samples of eluate were collected for scintillation counting.

(b) **Isolation of 28S and 18S rRNA.** Individual culture dishes were washed briefly three times with phosphate-buffered saline, and total cell RNA was extracted with acid guanidinium thiocyanate/phenol/chloroform (Chomczynski & Sacchi, 1987). The 18S and 28S RNA in samples (5–10 μ g) of total cellular RNA were separated under denaturing conditions by electrophoresis on 1.2% agarose gels + 2.2% formaldehyde (Maniatis *et al.*, 1982). Bands of 18S and 28S RNA were detected over u.v. light after ethidium bromide treatment, excised, dissolved by warming in 1 ml of water and counted for radioactivity in 5 ml of scintillation fluid.

(c) **Isolation of 40S and 60S ribosomal subunits.** Dishes were washed three times in phosphate-buffered saline, and the contents of three or four dishes were combined

by scraping off the dishes into a total volume of 3 ml of phosphate-buffered saline, which was transferred to a 10 ml conical plastic tube. Cells were pelleted by centrifugation at 1000 *g* for 5 min at 4 °C and then resuspended in 0.8 ml of buffer solution containing 130 mM-KCl, 0.5 mM-CaCl₂, 0.25 mM-sucrose and 10 mM-Tris, pH 7.6 (buffer 1), +0.1 ml of 10% (w/v) deoxycholate and 0.1 ml of 10% (w/v) Nonidet P40. After 10 min at 0 °C and centrifugation at 3000 *g* for 10 min at 4 °C, the supernatant was layered over a sucrose cushion and centrifuged at 100000 *g* for 16 h at 4 °C. The ribosomal pellet was resuspended by sonication for 30 s in 0.95 ml of buffer 1, to which GTP was added to a concentration of 1 mM. Then 50 μ l of 10 mM-puromycin and 20 μ l of 1 M-mercaptoethanol were added, and after 15 min incubation at 37 °C the samples were layered on to 10–30% (w/v) linear sucrose density gradients containing 10 mM-Tris, pH 7.6, 500 mM-KCl, 5 mM-MgCl₂ and 20 mM-mercaptoethanol and centrifuged for 7 h at 132000 *g* at 28 °C. Areas of the gradient containing 40S and 60S ribosomal subunits were located by measuring absorbance at 260 nm. These were dialysed against four changes of distilled water (5 litres each), freeze-dried, and dissolved in 1 ml of 0.3 M-NaOH at 37 °C for 30 min. Protein was precipitated by adding 0.3 ml of 2 M-HClO₄ and the RNA content and associated radioactivity were measured as described above.

Scintillation counting

Samples (maximum vol. 1 ml) were counted in 5 ml of NE265 scintillation fluid (N.E.N., Edinburgh, Scotland, U.K.). Counting efficiency was determined from quench curves obtained under similar counting conditions.

RESULTS

Effects of insulin, ibuprofen and indomethacin on labelling of intracellular pools and total cellular RNA

When cells were incubated for 6 h in the presence of 5 μ M-uridine, insulin stimulated total RNA accretion and uridine incorporation into RNA (+18% and +28% respectively; Table 1). Ibuprofen and indomethacin had no effect on the basal rates of RNA accretion or uridine incorporation but both non-steroidal anti-inflammatory drugs decreased the insulin-induced increase in accretion and synthesis of RNA.

In order to exclude the possibility that the observed effects of insulin on uridine incorporation were due to stimulation of transport leading to differences in precursor pool sizes, further experiments were carried out with a high concentration (1 mM) of lower-specific-radioactivity uridine in the culture medium. Under these labelling conditions, the recovery of total radioactivity from the intracellular nucleotide pool was examined at 30 min and 2, 4 and 6 h. By 2 h, labelling appeared to have reached a plateau value, which was maintained until 6 h. At 30 min, labelling of the free pool was approx. 60% of the plateau value (Table 2), and at none of the times studied were any effects of insulin or indomethacin observed. The specific radioactivities of the intracellular free uridine and UTP were not significantly different in control cultures (1014 ± 46 and 1196 ± 106 d.p.m./nmol respectively; means $\pm$ S.E.M.; $n = 4$). None of the three treatments (insulin, indomethacin, insulin + indomethacin) had a significant effect on the specific radioactivity of the UTP, giving values of

Table 1. Effects of indomethacin and ibuprofen on the stimulation of RNA synthesis and accretion by insulin

At 18 h after culture dishes were transferred to a serum-free medium, insulin (100 μ -units/ml) and/or indomethacin (50 μ M) or ibuprofen (100 μ M) were added for 6 h. Uridine (5 μ M) and [3 H]uridine (0.2 μ Ci/dish) were also present throughout this 6 h period. Values are means $\pm$ S.E.M. ($n = 6$). By Student's t test: **, ***, significantly different from control ($P < 0.01$, $P < 0.001$ respectively); ++, +++, significantly different from insulin alone ($P < 0.01$, $P < 0.001$ respectively).

Treatment	Total RNA (μ g/dish)	Uridine incorpora- tion (d.p.m./ μ g of RNA)
Control	66 $\pm$ 2	379 $\pm$ 23
Indomethacin (50 μ M)	67 $\pm$ 2	393 $\pm$ 23
Ibuprofen (150 μ M)	66 $\pm$ 2	422 $\pm$ 10
Insulin (100 μ -units/ml)	78 $\pm$ 1***	485 $\pm$ 5**
Insulin + indomethacin	65 $\pm$ 3++	400 $\pm$ 8+++
Insulin + ibuprofen	67 $\pm$ 1+++	422 $\pm$ 10++

1172 $\pm$ 114, 1018 $\pm$ 113 and 1140 $\pm$ 98 d.p.m./nmol respectively (means $\pm$ S.E.M.; $n = 4$). As a proportion of the total radioactivity recovered, UTP represented 7.1–7.8% at 4 h, and was also not significantly affected by insulin or indomethacin (Table 2). UTP was the major metabolite found, UDP represented only 2.3% of the total radioactivity recovered and was also unchanged by the various treatments (results not shown), and very little radioactivity was detected in the area corresponding to UMP. Thus neither insulin nor indomethacin appeared to change the rate of transport of uridine into the cells or the conversion of uridine into UTP. Nevertheless, at this higher uridine concentration (1 mM), insulin produced significant increases in uridine incorporation into RNA (+16% and +21% at 4 and 6 h respectively; Table 2), and the increases in uridine incorporation in response

to insulin at both these times were inhibited by indomethacin.

Effects on incorporation into rRNA

Further experiments were carried out in which incorporation specifically into rRNA was measured. In the first series of experiments total cellular RNA was extracted from cells after a 6 h labelling period, and 18S and 28S rRNA were separated by agarose-gel electrophoresis. In the presence of 1 mM-uridine the insulin-induced increases were 51% and 68% respectively in the 18S and 28S rRNA. Similar increases in response to insulin were seen in the two experiments with 5 μ M-uridine (Table 3). The stimulation of incorporation into both subunit species by insulin was inhibited by indomethacin at both uridine concentrations; the effect of ibuprofen was examined only in the presence of 1 mM-uridine, and was similar to that of indomethacin.

Isolation of the 40S and 60S ribosomal subunits by sucrose-density-gradient centrifugation after dissociation with puromycin produced similar results; both subunits showed considerably increased [3 H]uridine incorporation after a 6 h exposure of the cells to insulin (Table 4), and the effect of insulin on incorporation into both subunits was totally inhibited by indomethacin. To examine the purity of the two subunits, agarose-gel electrophoresis was performed and showed that the 40S fraction contained no 28S RNA and the 60S fraction contained no 18S RNA (results not shown).

DISCUSSION

Previous studies of RNA synthesis by using uridine incorporation have been complicated by problems of precursor-pool compartmentation and uridine transport. Insulin, for example, stimulated uridine entry into fibroblasts (Rozenfurt & Stein, 1977), and pool compartmentation was identified as a potential problem in the interpretation of results involving the labelling of RNA with pyrimidine precursors (Plagemann, 1971). In the present experiments uridine incorporation was

Table 2. Effects of insulin and indomethacin on the incorporation of [3 H]uridine into the intracellular pool and total RNA of L6 myoblasts

At 18 h after culture dishes were transferred to a serum-free medium; insulin (Ins; 100 μ -units/ml) and/or indomethacin (Ind; 50 μ M) were added for 6 h. Uridine (5 μ M) containing [3 H]uridine (0.2 μ Ci/dish) was also present throughout this 6 h period. Values for both intracellular free uridine and uridine incorporated into RNA are expressed as d.p.m. recovered per μ g of tissue RNA. Values are means $\pm$ S.E.M. ($n = 5$). By Student's t test: **, ***, significantly different from controls ($P < 0.01$, $P < 0.001$ respectively).

	Control	+ Ins	+ Ind	+ Ins + Ind
30 min incubation (d.p.m. free/ μ g of RNA)	384 $\pm$ 34	300 $\pm$ 28	321 $\pm$ 40	369 $\pm$ 20
2 h incubation (d.p.m. bound/ μ g of RNA)	58 $\pm$ 5	74 $\pm$ 12	64 $\pm$ 6	66 $\pm$ 4
(d.p.m. free/ μ g of RNA)	567 $\pm$ 57	583 $\pm$ 36	620 $\pm$ 35	612 $\pm$ 33
4 h incubation (d.p.m. bound/ μ g of RNA)	101 $\pm$ 5	117 $\pm$ 4**	99 $\pm$ 6	102 $\pm$ 7
(d.p.m. free/ μ g of RNA)	580 $\pm$ 25	627 $\pm$ 87	583 $\pm$ 63	555 $\pm$ 21
% as UTP	7.4 $\pm$ 0.8	7.1 $\pm$ 0.6	7.5 $\pm$ 0.6	7.8 $\pm$ 0.5
6 h incubation (d.p.m. bound/ μ g of RNA)	153 $\pm$ 9	186 $\pm$ 2***	146 $\pm$ 5	155 $\pm$ 2
(d.p.m. free/ μ g of RNA)	571 $\pm$ 30	613 $\pm$ 35	606 $\pm$ 19	576 $\pm$ 27

Table 3. Effects of insulin and indomethacin on uridine incorporated into 18S and 28S RNA separated by agarose-gel electrophoresis

Effects of insulin (100 μ -units/ml), ibuprofen (100 μ M) and indomethacin (50 μ M) on the incorporation of [3 H]uridine into RNA species were examined over a 6 h incubation period in the presence of 1 mM- or 5 μ M-uridine. Values from two experiments with 5 μ M-uridine are shown individually; values obtained with 1 mM-uridine are shown as means $\pm$ S.E.M. ($n = 5$). Significances of differences were determined by Student's t test by using paired values: significantly different from control, * $P < 0.05$; significantly different from insulin alone, † $P < 0.05$, †† $P < 0.01$.

Labelled with 1 mM-uridine	Uridine incorporated (d.p.m./ μ g of RNA loaded)	
	18S	28S
Control	18.3 $\pm$ 5.2	24.2 $\pm$ 4.5
Insulin	27.7 $\pm$ 8.0*	40.7 $\pm$ 8.1*
Indomethacin	15.0 $\pm$ 5.0	19.4 $\pm$ 6.6
Ibuprofen	10.2 $\pm$ 3.8	17.3 $\pm$ 4.3
Insulin + indomethacin	13.6 $\pm$ 6.4	21.5 $\pm$ 6.9††
Insulin + ibuprofen	15.9 $\pm$ 7.5†	21.4 $\pm$ 5.7††

Labelled with 5 μ M-uridine	Uridine incorporated (d.p.m./ μ g of RNA loaded)			
	1		2	
	18S	28S	18S	28S
Expt. no. ...				
Control	52	77	97	118
Insulin	80	137	191	210
Indomethacin	51	88	79	96
Insulin + indomethacin	37	47	102	124

investigated at two concentrations, 5 μ M and 1 mM. The higher concentration was used in an attempt to achieve rapid flooding of the intracellular uridine pools so as to preclude effects of insulin or the non-steroidal anti-inflammatory drugs on uridine transport or metabolism. With this high extracellular concentration of uridine, the labelling of the intracellular pools appeared to have reached a plateau by 2 h, whereas after only 30 min labelling the intracellular pool had attained approx. 60% of this plateau value. Neither insulin nor indomethacin had a significant effect on the incorporation of [3 H]uridine into the total intracellular pool at any of the times studied. Such findings are consistent with previous results showing that in most cell types there is a rapid equilibration between various uridine pools (Khym *et al.*, 1978).

Analysis of cell extracts showed that, as found in the mammary gland (Turkington, 1970), UTP was the major metabolite of uridine in the L6 myoblasts, and represented 7–8% of the total intracellular radioactivity; no significant effect of insulin or indomethacin on labelling of UTP was found.

To exclude the possibility that 1 mM-uridine might itself influence the metabolism of RNA, similar experiments were performed with 5 μ M-uridine. These experiments produced similar results, again demonstrating that insulin-stimulated RNA metabolism was blocked by indomethacin. Thus, under the conditions that we used, there is no evidence of effects of insulin or the prostaglandin-inhibitory drugs which would compromise the interpretation that insulin stimulates rRNA synthesis in L6 cells.

Two methods were used to investigate the effects of insulin and the non-steroidal anti-inflammatory drugs on the incorporation of uridine into ribosomes. Total RNA extraction followed by agarose-gel electrophoresis measured incorporation of uridine into all the 18S and 28S RNA regardless of whether these were associated with ribosomes or represented a pool of newly synthesized RNA species, a situation which has been observed after myotube formation in myoblast cultures (Krauter *et al.*, 1980). The second approach, separation of the 40S and 60S subunits by sucrose-density-gradient centrifugation, should preclude the presence of free 18S and 28S RNA, and so should give a measure of incorporation into rRNA assembled into subunit material.

Considered together, the very similar results obtained by the two methods provide clear evidence that insulin stimulates synthesis of both rRNA species, that the RNA is subsequently incorporated into subunits, that the effect on the two subunits is similar and that inhibitors of the enzyme cyclo-oxygenase inhibit this stimulation. This is consistent with previous work which has shown that insulin stimulates ribosome synthesis in muscle from diabetic rats (Ashford & Pain, 1986) and rRNA synthesis in the mammary gland (Turkington, 1970) and in fibroblasts in culture (Hershko *et al.*, 1971). Comparison of the results obtained from the total RNA (Tables 1 and 2) and rRNA (Tables 3 and 4) shows considerable differences between the total RNA and rRNA, both in the percentage increase induced by insulin and in the specific radioactivity. The effect of insulin on rRNA appears to be proportionately greater, as labelling of 18S and 28S rRNA was increased by 50–70% in response

Table 4. Effects of insulin and indomethacin on uridine incorporation into 40S and 60S ribosomal subunits separated by sucrose-density-gradient centrifugation after dissociation with puromycin

Effects of insulin (100 μ -units/ml) and indomethacin (50 μ M) on the incorporation of [3 H]uridine into ribosomal subunits were examined over a 6 h incubation period in the presence of 1 mM- or 5 μ M-uridine. Values from two experiments with 5 μ M-uridine are shown individually. Values obtained with 1 mM-uridine are from a single experiment.

Labelled with 1 mM-uridine	Uridine incorporated (d.p.m./ μ g of RNA)	
	40S	60S
Control	35.5	36.2
Insulin	48.3	52.6
Indomethacin	29.9	27.5
Insulin + indomethacin	32.1	32.0

Labelled with 5 μ M-uridine	Uridine incorporated (d.p.m./ μ g of RNA)			
	1		2	
	40S	60S	40S	60S
Expt. no. ...				
Control	61.4	73.4	87.8	94.2
Insulin	84.9	100.6	124.0	134.0
Indomethacin	61.6	50.6	68.6	66.6
Insulin + indomethacin	55.6	63.0	67.2	71.1

to 6 h incubation with insulin, whereas labelling of the total RNA was increased by only 18% over the same period. However, the higher specific radioactivity of the total RNA suggests that non-ribosomal RNA species have a substantially higher rate of synthesis than the rRNAs. This was supported by the fact that, although the 18S and 28S rRNA species were the major bands present after agarose-gel electrophoresis, representing 80% of the RNA recovered, they contained only approx. 40% of the total radioactivity. Thus in the unstimulated cell much of the incorporation of uridine is the result of rapid turnover of mRNA and only a small proportion is due to the synthesis of the major (ribosomal) component, whereas after addition of insulin rRNA synthesis is stimulated and the effect of the hormone on mRNA synthesis is small.

Recently insulin was reported to stimulate rRNA synthesis in mouse myoblasts by increasing ribosomal-DNA transcription (Hammond & Bowman, 1988). However, these results were achieved with an insulin concentration of 4 μ g/ml, i.e. approx. 1000 times the physiological concentration and 500 times the concentration used in the present experiments. Whereas the results of Hammond & Bowman (1988) may be due to an effect via the insulin-like-growth-factor receptor (Surmacz *et al.*, 1987), the present results, obtained with an insulin concentration only approximately twice that found in fully fed rats, are most likely to be due to insulin acting through the insulin receptor itself.

Indomethacin has been shown previously to inhibit the production of prostaglandin F_{2x} by 80% in L6 myoblasts and to inhibit the ability of insulin to stimulate RNA synthesis (Palmer & Bain, 1989). The decrease in total RNA content of normal and hypertrophying muscle by fenbufen (McMillan *et al.*, 1988) has implicated the

eicosanoids in the control of RNA synthesis *in vivo*. The present paper extends these observations by demonstrating an effect of two inhibitors of prostaglandin synthesis specifically on rRNA. The results presented here show little evidence that either indomethacin or ibuprofen had effects on the basal rate of rRNA synthesis, and suggest that a prostaglandin or other eicosanoid metabolite of arachidonic acid is involved not in the maintenance of basal synthesis but in the signal mechanisms whereby insulin stimulates RNA synthesis in myoblasts. Previous studies have implicated metabolites of arachidonic acid in the short-term control of protein synthesis (Smith *et al.*, 1983; Reeds & Palmer, 1983). Thus the eicosanoids may be involved in control of both translational activity and rRNA synthesis.

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