Biochemical Pharmacology, Vol. 44, No. 7, pp. 1291–1296, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

THE EFFECTS OF IBUPROFEN ENANTIOMERS ON HEPATOCYTE INTERMEDIARY METABOLISM AND MITOCHONDRIAL RESPIRATION

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(*Received* 22 *April* 1992; *accepted* 14 *July* 1992)

Abstract—*In vivo* and *in vitro* (−)*R*-ibuprofen is inverted to the (+)*S* antipode via stereoselective formation of an *R*-ibuprofenyl-CoA intermediate. In this study the effects of (−)*R*- and (+)*S*-ibuprofen on metabolism and respiration were studied using isolated rat hepatocytes and mitochondria. *R*-Ibuprofen significantly increased the lactate to pyruvate ratio, perturbed mitochondrial ketogenesis as evidenced by alterations in the β-hydroxybutyrate to acetoacetate ratio and uncoupled mitochondrial oxidative phosphorylation. In addition, substantial dose- and time-dependent sequestration of reduced CoA (CoASH) occurred in the presence of the *R* enantiomer. Similarly, *S*-ibuprofen altered both the cytosolic and mitochondrial redox states although the magnitude of the effect was substantially less than that observed with the *R* enantiomer. In contrast to *R*-ibuprofen, *S*-ibuprofen did not uncouple oxidative phosphorylation or sequester hepatocyte CoASH. It is proposed that the perturbations observed in hepatocyte intermediary metabolism and mitochondrial function are attributable to a combination of the direct effects of *R*-ibuprofen *per se* and the sequestration of CoASH as *R*-ibuprofenyl-CoA during the process of chiral inversion. On the basis of these results, *R*-ibuprofen should be considered more in terms of metabolism to a reactive acyl-CoA intermediate rather than as a pro-drug for the pharmacologically active *S*-enantiomer.

Ibuprofen, a non-steroidal anti-inflammatory 2-arylpropionate, possesses a chiral centre and therefore exists as (−)*R* and (+)*S* enantiomers. It is administered to humans as a racemate and *in vivo* (−)*R*-ibuprofen is inverted to the pharmacologically active (+)*S* antipode via a CoA thioester intermediate. This reaction is reportedly catalysed by long chain fatty acid CoA ligase [1–2], an enzyme predominantly involved in the activation of fatty acids to the corresponding acyl-CoAs. Formation of an *R*-arylpropionyl-CoA thioester intermediate provides an explanation for both the stereospecific inhibition of triacylglycerol synthesis by *R*-fenoprofen *in vitro* [3] and the incorporation of *R*-ibuprofen into adiopose tissue triglycerides *in vivo* [4].

CoA plays a pivotal role in intermediary metabolism and maintenance of the [acyl-CoA]:[CoASH] ratio in both the cytosol and mitochondrial matrix depends on the relative balance between cellular reactions which acylate and release CoASH. Generation of *R*-ibuprofen-CoA intracellularly may well have the potential to interfere with many CoA-dependent reactions. *R*-Ibuprofen has previously been used to study the mechanism of chiral inversion in isolated hepatocytes [5] and the stereoselective effects of ibuprofen on β-oxidation in rodent mitochondria [6]. To date however there are no data on the effects of ibuprofen enantiomers on either hepatocyte intermediary metabolism or mitochondrial respiration.

Therefore, the aim of the present study was to examine using isolated hepatocytes and mitochondria the stereoselective effects of *R*- and *S*-ibuprofen on intracellular CoASH levels, pyruvate oxidation, mitochondrial ketogenesis and oxidative phosphorylation.

MATERIALS AND METHODS

Chemicals. (−)*R*- and (+)*S*-ibuprofen were generously supplied by the Boots Co. (Nottingham, U.K.). The optical purities were 97.3% and 98.4% for *R*- and *S*-ibuprofen, respectively [7]. β-NADH (disodium salt, Grade III), βNAD (Grade III), ADP (sodium salt) and collagenase (Type IV) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.); succinic acid (disodium salt) from Calbiochem-Behring Corp. (La Jolla, CA, U.S.A.); and 3-hydroxybutyrate dehydrogenase (Grade II) and lactate dehydrogenase (from hog muscle in glycerol) from Boehringer Mannheim (Germany). All other reagents were of the highest grade commercially available.

Animals. Male outbred black hooded Wistar rats (250–350 g) were housed under standard conditions of temperature and light and allowed food and water *ad lib.*

Preparation and incubation of hepatocytes. Hepatocytes were isolated as described previously [8] and suspended in Krebs–Henseleit glucose (0.5%) buffer

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‡ Abbreviations: CoASH, coenzyme A (reduced); DMSO, dimethyl sulphoxide; ALT, alanine aminotransferase; RCR, respiratory control rate.

(pH 7.5) (2×10^6 cells/mL). Cell viability was determined using Trypan blue (0.25% w/v) and only cell preparations with an exclusion index of >90% were utilized. The yield of hepatocytes ranged from 3.8 to 5.2×10^8 cells/liver. Hepatocytes (6×10^6 cells) were incubated (37°) in conical flasks (25 mL) and gassed continuously with 95%O_2/5% CO_2. All flasks were pre-incubated for 5 min prior to the addition of either *R*- or *S*-ibuprofen (1, 1.5 or 3 mM, final concentration). The substrate was added in a minimal volume of dimethyl sulphoxide (DMSO) (20 μL) and control cells received vehicle only.

Hepatocyte assays. At various times (30–240 min) aliquots of the hepatocyte incubations were collected and prepared for analysis of metabolites and alanine aminotransferase as described previously [9]. Using an automated centrifugal analyser (Cobas-Bio, Roche Switzerland) acetoacetate, β-hydroxybutyrate, lactate and pyruvate were all analysed separately on the same sample by changing at the appropriate time the reagents and enzymes specific to each metabolite, i.e. 3-hydroxybutyrate dehydrogenase and lactate dehydrogenase. Prior to each run the equipment was calibrated using either quality control sera or primary standards. CoASH was determined on an aliquot (0.2 mL) of the supernatant used for the metabolite assays. Dithiothreitol (0.6 mg) and ether (1 mL) were added to each sample and the tubes capped and agitated vigorously. The organic layer was removed after centrifugation at 500 *g* for 10 min and the aqueous phase adjusted to pH 8.4–8.6 and assayed for CoASH as described previously [10].

Isolation and incubation of mitochondria. Liver mitochondria were isolated with minor procedural modifications [11, 12]. The final mitochondrial pellet was resuspended (1 mg/mL) in ice-cold respiration medium containing 250 mM sucrose, 20 mM KH_2PO_4, 5 mM $MgCl_2$, 20 mM KCl, 20 mM Tris and 1 mM EDTA. Rotenone (9 μg) was added to prevent accumulation of oxaloacetate which inhibits succinate dehydrogenase. After equilibration of the air saturated medium (4.9 mL, pH 7.4) at 37° 2 mM succinate was added followed 1 min later by the addition of either ADP (0.2 mM) (untreated mitochondria) or *R*- or *S*-ibuprofen (50–200 μM in DMSO). In the latter situation ADP was added 1.5 min after the xenobiotic. Mitochondrial respiration in the presence of ADP (State 3) and following depletion of ADP (State 4) was measured using a Clark type oxygen electrode (Rank Brothers, Cambridge, U.K.) equipped with a temperature-regulated water bath. Respiratory control rate (RCR) and ADP:O ratio were calculated as described by Estabrook [13]. Mitochondrial protein was assayed according to Lowry *et al.* [14].

Statistical analysis. The statistical significance of the difference between groups was determined by Student's *t*-test with Bonferroni correction [15].

RESULTS

Hepatocyte viability

Hepatocytes treated with DMSO only exhibited a consistently low level of alanine aminotransferase (ALT) leakage (14.2 ± 4.2%) over the 4 hr of incubation. No significant increase in ALT release was observed in the presence of either 1 mM *R*- or *S*-ibuprofen; however, addition of 1.5 mM resulted in a small but significant increase ($P < 0.01$) in enzyme leakage at 30 min: *R*, 16.7 ± 2.1 and *S*, 16.9 ± 1.3% (mean ± SD). At a concentration of 3 mM both enantiomers produced a significant increase in ALT leakage ($P < 0.001$) at all times studied (30–240 min). The total loss of ALT at 240 min was comparable for *R*- and *S*-ibuprofen: 28.7 ± 3.7 and 27.3 ± 4.5% (mean ± SD N = 7), respectively.

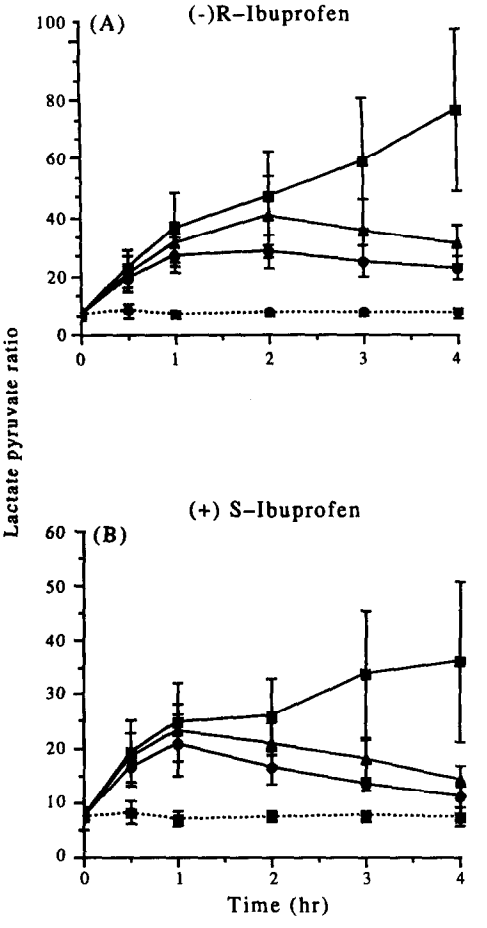

Fig. 1. Lactate to pyruvate ratio was measured in hepatocytes exposed to either vehicle (DMSO) (●----●) or 1 mM (●), 1.5 mM (▲) or 3 mM (■) *R* (A) or *S* (B)-ibuprofen. Data are presented as means ± SD of seven cell preparations. All values are statistically significantly different from the respective control values at $P < 0.001$.

Lactate to pyruvate ratio (Fig. 1)

In hepatocyte suspensions incubated in the absence of ibuprofen enantiomers the concentration of both lactate and pyruvate remained constant over the 4 hr: 3.59 ± 1.48 and 0.46 ± 0.16 mM (mean ± SD, N = 7), respectively. In contrast, cells incubated in the presence of *R*-ibuprofen exhibited a dose-dependent accumulation of lactate with only a minor

elevation of pyruvate concentration. This resulted in a substantial increase in the lactate to pyruvate ratio (Fig. 1A). Although incubation with the (+)*S* enantiomer also significantly increased the lactate to pyruvate ratio the magnitude of the increase was substantially less than that observed with the equivalent concentration of (−)*R*-ibuprofen (Fig. 1B), e.g. for 3 mM, 76.6 ± 27.4 and 36 ± 14.9 mean ± SD, N = 7) at 240 min for *R*- and *S*-ibuprofen, respectively.

β-Hydroxybutyrate to acetoacetate ratio

The ratio of the ketone bodies β-hydroxybutyrate and acetoacetate in hepatocyte suspensions not exposed to ibuprofen remained constant for the duration of the experiment, 0.61 ± 0.03 (N = 42). In the presence of all concentrations of *R*-ibuprofen the ratio was maximally increased at 60 min but thereafter decreased such that at 240 min the ratio was similar to control cells with 1.5 mM and was significantly reduced ($P < 0.001$) in the presence of 3 mM *R*-ibuprofen (Fig. 2A).

Similarly, with the *S* enantiomer a significant increase ($P < 0.01$) in the ratio was evident for all concentrations at 60 min. Subsequently, in the presence of both 1 and 1.5 mM *S*-ibuprofen the ratio declined gradually but remained significantly elevated whilst in the presence of 3 mM *S*-ibuprofen; the ratio had returned to control values by 240 min (Fig. 2B). This profile was explained by an apparent two phase response. During the first 60 min the concentration of β-hydroxybutyrate was increased compared with control incubations (0.13 ± 0.04 mM); however, the acetoacetate levels were decreased (0.11 ± 0.03 mM). Continued incubation for a further 180 min resulted in a further increase in β-hydroxybutyrate levels relative to the control incubations while the concentration of acetoacetate increased such that it was either quantitatively similar to or exceeded the corresponding value for β-hydroxybutyrate.

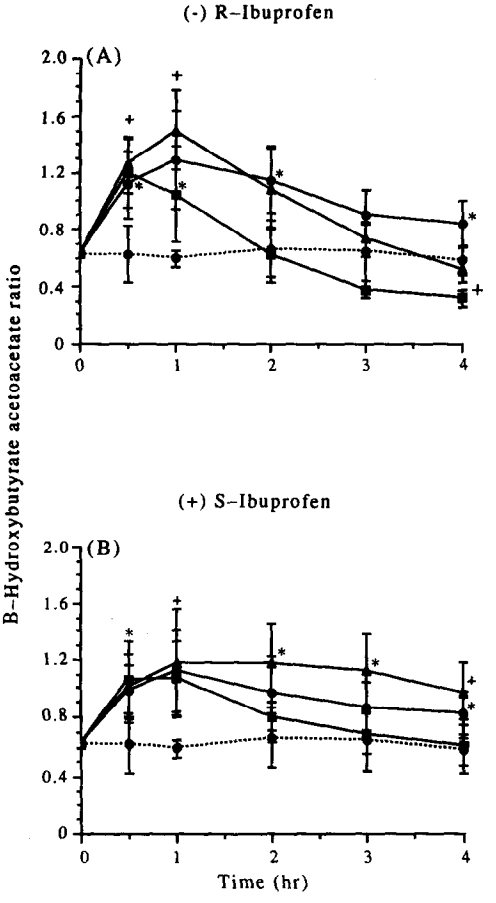

Fig. 2. The concentration of β-hydroxybutyrate and acetoacetate was determined following incubation with either (●) 1 mM, (▲) 1.5 mM or (■) 3 mM *R* (A) or *S* (B)-ibuprofen. Control incubations received (DMSO) (●----●) only. Data are presented as means ± SD of seven hepatocyte preparations. β-Hydroxybutyrate: acetoacetate ratio immediately prior to addition of either vehicle or drug was 0.69 ± 0.04. Statistically significant difference from control values denoted by *$P < 0.01$ and +$P < 0.001$.

Hepatocyte CoASH content

No significant decrease in the concentration of CoASH was observed in the presence of (+)*S*-ibuprofen. However, following incubation with the *R* enantiomer, CoASH content was significantly decreased ($P < 0.05$) in a dose-dependent manner from an initial value of 2.13 ± 0.27 nmol/10^6 cells (Fig. 3A). The time course for depletion of hepatocyte CoASH by 1.5 mM *R*-ibuprofen is shown in Fig. 3B.

Mitochondrial respiration and oxidative phosphorylation

In the absence of ibuprofen isolated mitochondria had a succinate-supported RCR of 4.3 ± 0.48 and an ADP:O ratio of 1.7 ± 0.2 (mean ± SD, N = 5) indicating tightly coupled mitochondrial respiration. Addition of either *R*- or *S*-ibuprofen (50–200 μM) reduced succinate-supported State 3 respiration and significantly reduced ($P < 0.001$) the RCR. This loss of RCR was accompanied by a stimulation of State 4 respiration in the presence of 100 and 200 μM *R*-ibuprofen. The ADP:O ratio was significantly reduced ($P < 0.05$) by all concentrations of *R*-ibuprofen; however, this effect was not observed with the lower concentrations of *S*-ibuprofen, i.e. 50 and 100 μM (Table 1). In contrast to the *R* enantiomer, *S*-ibuprofen did not significantly suppress State 3 or stimulate State 4 respiration.

DISCUSSION

In hepatocytes, the lactate–pyruvate system provides the most reliable value for cytosolic NAD^+/NADH while the use of the β-hydroxybutyrate:acetoacetate ratio is a measure of the mitochondrial redox state [16]. In normal cells, coupling of cytosolic and mitochondrial processes ensures that NADH does not accumulate and that the difference in redox potential between the two cellular compartments is maintained. Hepatocytes incubated in the presence of *R*-ibuprofen exhibited a dose-dependent accumulation of lactate and thus

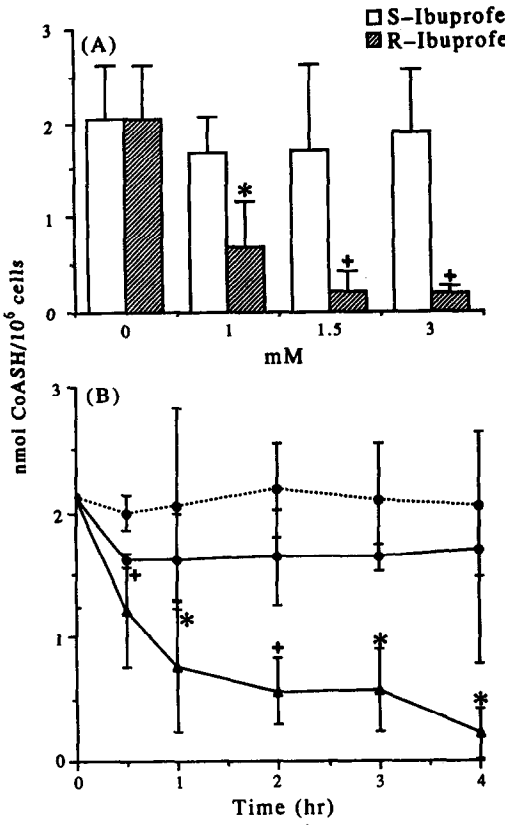

Fig. 3. CoASH was determined in hepatocytes incubated with either *R*- or *S*-ibuprofen, 1, 1.5 or 3 mM, for 240 min (A). In addition, hepatocytes were exposed to 1.5 mM *S* (●) or *R* (▲)-ibuprofen for 30–240 min. Control cells were incubated in the presence of DMSO only (●----●) (B). Data are presented as means ± SD (N = 5) and statistically significant difference from control values denoted by *P < 0.05 and +P < 0.01.

an increase in the lactate:pyruvate ratio. This increased ratio reflected an accumulation of carbon at the bottom of the glycolytic pathway and therefore an increase in the reducing equivalents in the cytosol. It is interesting to note that *S*-ibuprofen also increased both the lactate:pyruvate and β-hydroxybutyrate:acetoacetate ratios although, the overall effect was substantially less than that observed with the *R* enantiomer. Deficiencies in mitochondrial oxidative phosphorylation will also alter the NAD^+:NADH ratio and as a consequence the cytosolic lactate:pyruvate ratio increases. Under these circumstances the oxidative capacity of the liver cell is lessened and in order for metabolism to continue utilization of acetoacetate is promoted when alternative sources for acetyl-CoA such as oxidation of pyruvate and fatty acids are not sufficient. This sequence of events provides an explanation for the observation of decreased acetoacetate levels over the first 60 min of incubation with *R*-ibuprofen. Over the subsequent 180 min, the concentration of both ketone bodies increased reflecting an increase in the NAD^+:NADH ratio, possibly occurring as a result of inhibition of β-oxidation of fatty acids [6, 17].

An additional consideration, however, is the fact that chiral inversion of *R*-ibuprofen to the *S* antipode via a CoA intermediate [18] occurs in isolated hepatocytes [5]. In this study, CoASH concentration was reduced by 43% at 30 min and 90% at 240 min in the presence of 1.5 mM *R*-ibuprofen. CoA is an essential component of intermediary metabolism and sequestration by *R*-ibuprofen may further explain the perturbations observed in both pyruvate and fatty acid oxidation. An additional explanation therefore for the increased utilization of acetoacetate observed in this study may be diminished formation of long chain fatty acid acyl-CoAs due to the extramitochondrial sequestration of CoASH as a result of *R*-ibuprofen-CoA thioester formation. Sequestration of CoASH has been evoked to explain the inhibition of hepatocyte gluconeogenesis and lipogenesis by benzoic acid and other structurally related carboxylic acids [19]. Although a diminution in the concentration of CoASH was observed in the

Table 1. Mitochondrial respiration and oxidative phosphorylation

	State 3	State 4	RCR	ADP:O
	(nmol O_2 consumed/mg/min)			
Control	163.6 ± 18.2	39.9 ± 8.4	4.3 ± 1.1	1.7 ± 0.2
DMSO	158.6 ± 5.8	48.3 ± 4.3	4.4 ± 0.3	1.7 ± 0.1
(−)*R* 50 μM	132.6 ± 25.5	57.0 ± 6.7	2.54 ± 0.6§	1.3 ± 0.2*
100 μM	137.1 ± 23.5	61.3 ± 7.7†	2.1 ± 0.3§	1.3 ± 0.2*
200 μM	117.6 ± 23.8†	71.8 ± 14.3†	1.7 ± 0.4§	1.2 ± 0.2‡
(+)*S* 50 μM	135.1 ± 34.8	55.3 ± 8.7	2.4 ± 0.5§	1.6 ± 0.3
100 μM	129.2 ± 28.5	59.8 ± 11.4	2.1 ± 0.4§	1.4 ± 0.3
200 μM	123.6 ± 36.3	57.3 ± 8.9	1.9 ± 0.5§	1.2 ± 0.2†

Effect of ibuprofen enantiomers on isolated rat liver mitochondria. Oxygen consumption was measured in the presence of 2 mM succinate. See text for experimental details.
The data are tabulated as means ± SD (N = 5).
Significantly different from control, *P < 0.05, †P < 0.02, ‡P < 0.01, §P < 0.001.
Final DMSO concentration = 0.22%.

presence of the *S* enantiomer, this may be accounted for by a 1.6% impurity with the optical antipode.

Uncoupling of mitochondrial oxidative phosphorylation *in vitro* is a relatively common property of several classes of anti-inflammatory drugs [20] and a previous study has demonstrated no difference in the uncoupling activities of the enantiomers of clidanac [21]. *R*- and *S*-ibuprofen both significantly reduced the respiratory control ratio at concentrations 20-fold less than those producing perturbations in the cytosolic and mitochondrial redox states. In contrast to the *S* enantiomer, *R*-ibuprofen significantly depresssed State 3 and stimulated State 4 respiration and inhibited the phosphorylation of ADP, indicating an uncoupling of mitochondrial oxidative phosphorylation. The uncoupling activity of racemic benoxaprofen [22] and ibuprofen [23] has been reported previously but to our knowledge this is the first report of differences in the uncoupling activity of the enantiomers of ibuprofen.

Although the situation is extremely complex with regard to identifying the initiating event, the results of this study can be explained on the basis of the stereoselective effects of the *R* enantiomer. *R*-Ibuprofen competitively inhibits palmitoyl-CoA formation [24] which consequently perturbs β-oxidation of palmitic acid [6]. This in turn diminishes the flow of electron equivalents to the respiratory chain. In addition, *R*-ibuprofen *per se* uncouples oxidative phosphorylation which may lead to a reduction in cellular ATP levels and consequentially cell death. The overall compounding of these effects is manifested initially by marked changes in both the cytosolic and mitochondrial redox states. Superimposed on this sequence of events is the additional insult of sequestration of cytosolic CoASH due to *R*-ibuprofenyl-CoA formation. Formation of this intermediate may then further contribute to the effects observed on pyruvate oxidation and mitochondrial ketogenesis as reported in this study. In contrast, *S*-ibuprofen which does not undergo chiral inversion and neither competitively inhibits palmitoyl-CoA formation [24] nor significantly inhibits palmitate oxidation [6] allows continued flow of electron equivalents to the respiratory chain which is not uncoupled in the presence of the *S* enantiomer. The results of this study demonstrate that *R*-ibuprofen produces substantially greater perturbations of hepatocyte cytosolic and mitochondrial redox states than the *S* enantiomer. These effects may in part be attributable to metabolic chiral inversion of *R*-ibuprofen and thus the data presented lends further support to the clinical use of enantiomerically pure *S*-arylpropionates.

It is interesting to note that in one report of acute intoxication with racemic ibuprofen the patient presented with a serum concentration of 897 μM and thereafter developed metabolic acidosis and acute liver cell injury [25]. Recent studies using primary cultures of rat hepatocytes and racemic ibuprofen have demonstrated cytotoxicity at concentrations greater than 1 mM [26, 27] and in this study moderate leakage of ALT was observed at the higher concentrations. However, the relationship between the mechanism of acute hepatotoxicity *in vivo* and the observations of cytotoxicity *in vitro* as reported in this and other studies remains to be established.

Acknowledgements—This research was funded by the National Health and Medical Research Council of Australia. The authors acknowledge the technical assistance of M. Cassidy and the Department of Clinical Biochemistry for use of the auto-analysers.

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