Effects of a thyromimetic on apolipoprotein B-100 in rats

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ABSTRACT

We have studied the effects of a cardiac sparing thyromimetic, CGS 23425, on postprandial levels of triglycerides, abundance of apolipoprotein B (apo B) protein and hepatic apo B mRNA expression in rats. When compared with control rats, triglyceride clearance was significantly accelerated by treatment with CGS 23425. A full return to baseline values was achieved within 8 h after ingesting a large quantity of fat, as compared to >24 h in control animals. The abundance of apo B-100 protein in CGS 23425-treated hyperlipidemic rats decreased in a dose-dependent manner, but levels of apo B-48 were not significantly affected. Like l-triiodothyronine (l-T₃), treatment with 30 µg/kg CGS 23425 for 6 or 9 days decreased the levels of apo B-100 protein by 80% and 40% respectively. This change was paralleled by a 27% reduction in hepatic apo B-100 mRNA. To investigate a potential mechanism of CGS 23425 action, we measured in vitro apo B mRNA editing activity in hepatocellular extract from control or CGS 23425-treated rats. Treatment with CGS 23425 increased activity of the hepatic apo B-100 editosome, apobec-1. In human hepatoma cells which lack apobec-1 activity, apo B-100 mRNA levels remained the same in cells treated with or without the agent. In summary, these observations show that CGS 23425 decreases the levels of apo B-100 in rats. This action of CGS 23425 involves apo B-100 mRNA editing activity.

Journal of Molecular Endocrinology (2000) 25, 299–308

INTRODUCTION

Ischemic heart disease (IHD) is the number one cause of premature death in Western societies (Gordon & Kannel 1972, Mann 1977). Hypercholesterolemia arising from dietary indiscretion is a major risk factor underlying this deadly disease (Mann 1977). Circulating cholesterol is carried by a variety of particles including low density lipoprotein (LDL) and chylomicron. Abnormally high levels of LDL cholesterol are associated with increased risk for developing IHD (Cullen & Assmann 1997, Lamarche & Lewis 1998). Apolipoprotein B (apo B) is the structural component of chylomicrons and triglyceride-rich very low density lipoproteins (VLDL), an immediate precursor of LDL (Sigurdsson et al. 1975). There are two forms of apo B, the full-length translation product, apo B-100, and a shorter form, apo B-48, corresponding to the amino-terminal 48% of the full-length protein (Kane 1983). In humans, the small intestine produces both apo B-100 and apo B-48 while the liver only produces apo B-100 (Glickman et al. 1986). However, both forms are normally present in mouse and rat liver (Greeve et al. 1993). Apo B-48 is derived from apo B-100 mRNA via a single post-transcriptional cytidine deamination that modifies the CAA codon encoding Gln-2153 and changes it to UAA, a stop codon (Powell et al. 1987, Teng et al. 1993). This modification interrupts the normal translation of apo B-100 mRNA at codon 2153 and thus gives rise to the truncated isofrom apo B-48. l-Tri-iodothyronine (l-T₃) and its analogs lower cholesterol and enhance the conversion of apo B-100 mRNA to the apo B-48 form in rat liver (Davidson et al. 1988a,b, 1990). Although these beneficial effects of l-T₃ on lipoprotein metabolism
are well known, the hormone cannot be used in the clinical setting of euthyroid individuals because it has undesirable cardiac effects. A novel thymimetic, CGS 23425, has no cardiotoxicity at a dose 4000-fold above that required to lower LDL cholesterol in a hypercholesterolemic rat (Taylor et al. 1997). CGS 23425 increased LDL-receptor numbers in Hep G2 cells and apo AI levels in the rat (Taylor et al. 1997). The latter effect was a result of increased apo AI gene transcription. In this study, we have examined the effects of CGS 23425 on the levels of triglyceride, apo B protein and apo B mRNA in rats. Additionally, a potential mechanism by which CGS 23425 exerts its action was tested in cytosolic extracts from rat liver.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing between 200 and 300 g, were housed under standard conditions of temperature and light with free access to water. Animals were handled in accordance with guidelines established by the NIH and the local animal ethics committee. For the postprandial triglyceride clearance study, rats were treated orally by gavage with water alone or aqueous CGS 23425 at 30 µg/kg, once daily for 7 consecutive days. Animals were fasted for 18 h then given an oral fat load consisting of 320 mg corn oil and 430 mg sucrose in a volume of 0·5 ml/rat. Blood was collected prior to the fat load and at 2, 4, 8, and 24 h postprandially. Plasma was prepared and triglyceride levels were determined with a diagnostic reagent kit (Sigma Chemical Co., St Louis, MO, USA) on a Bio-Mek workstation. Effects of CGS 23425 on rat apo B protein and mRNA levels were studied in three groups of animals: (i) cholesterol-fed hyperlipidemic animals maintained on a Purina chow diet supplemented with 1·5% cholesterol and 0·5% cholic acid (fat-fed) for 14 days before experiments; (ii) hyperthyroid rats prepared by intraperitoneal injection of 1·T3 (3,5,3′-tri-iodo-L-thyronine) at 15 µg/100 g body weight for 7 days; and (iii) rats rendered hypothyroid by 0·025% methimazole (Sigma) in the drinking water for at least 3 weeks. The group of fat-fed, euthyroid rats were treated orally by gavage with water or a solution of CGS 23425 at the doses indicated for 0, 6, and 9 consecutive days. Blood was collected by cardiac puncture under CO2 anesthesia into 5% EDTA. After the last dose, the animals were fasted for 18 h before being killed. Blood and liver were then collected for the studies outlined below.

Western-blot analysis of apo B

Plasma was prepared by centrifugation, and samples were diluted 1/25 in 10 mM Tris–EDTA (TE), pH 7·5. Five microliters of the diluted sample were added to 15 µl SDS loading buffer (5% SDS, 10% glycerol, 1·5% dithiothreitol (DTT), 1% 2-mercaptoethanol, 0·02% bromophenol blue in 0·12 M Tris, pH 6·8) and boiled before separation by electrophoresis on a 5–8% gradient SDS-polyacrylamide gel. The separated proteins were transferred onto PVDF membrane (Millipore Corp., Bedford, MA, USA) by electroblotting at 30 V for 18 h. The membranes were incubated in blocking buffer (0·3% Tween-20 (TTBS) and 10% (w/v) skimmed milk powder in TBS (10 mM Tris–HCl, pH 7·5, 150 mM NaCl)) for 1 h followed by liberal washes with TBS and then exposed to rabbit anti-apo B-48/100 antibody (Biodesign, Kennebunk, ME, USA) at 1:1000 in blocking buffer for 1 h at room temperature. After further washing and exposure to donkey anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Amersham, Oakville, OT, USA) at 1:5000 for 1 h, immunoreactive proteins were detected by chemiluminescent detection according to the manufacturer’s instructions (ECL, Amersham). Visualization of rat apo B-100 and apo B-48 was performed within 1 min. The relative concentration of apo B-100 to apo B-48 was assessed by video-assisted densitometry.

Preparation of cDNA and polymerase chain reaction

cDNA synthesis by reverse transcription polymerase chain reaction (RT-PCR) was performed using a method described previously (Davidson et al. 1988a) with several modifications. Total cellular RNA from liver isolated using procedures described previously (Barrera-Hernandez et al. 1996) was further purified by extracting with phenol/chloroform and precipitated with ethanol. Three micrograms total RNA were reverse transcribed using 200 U Moloney murine leukemia virus (MMLV) reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) in a buffer containing a final concentration of 75 mM KCl, 50 mM Tris, pH 8·3, 3 mM MgCl2, 10 mM DTT, 100 pmol random hexamers, 0·5 mM dNTPs, and 40 U RNAsin (Promega, Madison, WI, USA). After first strand cDNA synthesis, a 274 base pair fragment was amplified by means of the PCR. The following oligonucleotide primers were constructed containing the coding (PCR1) and non-coding (PCR3) sequences flanking nucleotide 6666 of apo B cDNA:

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\text{PCR1:} 5' - \text{CTAGAGCCAGCGCCTGGAGG} - 3' \\
\text{PCR3:} 5' - \text{CCCGTCCATTCACAGGAGCC} - 3'
\]
PCR1, ATCTGACTGGGAGAGACAAGTAG at 6512; PCR3, CACGGATGTGATCTG-TTCG TCAAGC at 6786 (Davidson et al. 1988a). The primers for amplification from the control rat β-actin sequences (Nusel et al. 1983) were: coding, CATGAAGTGACGTTGACATCC; and non-coding, TAACAGTCC-GCCTAGAAGCAATT TT GCG. PCR was conducted using 1 µM of each primer and 5 U Taq polymerase (Pharmacia, Morgan, Quebec, Canada) with final concentrations of 10 mM Tris, pH 9-0, 1-5 mM MgCl2, 50 mM KCl, 0-2 mM dNTPs and 2 µl cDNA. The template was denatured at 94 °C for 30 s, annealed at 55 °C for 1 min, and extended at 72 °C for 90 s, for a total of 22, 24, 25, 26 or 28 cycles with a final 10 min extension at 72 °C. After amplification, one-tenth of the PCR products were analyzed by 2-5% agarose electrophoresis to confirm the size of the amplified product and subsequently blotted onto nitrocellulose membrane (MSI, Westboro, MA, USA) in 0-4 M NaOH. Membranes were rinsed briefly in 2 × SSC and baked at 80 °C for 60 min. The apo B-100 or -48 bands were normalized to the β-actin signal.

**Differential hybridization**

The following oligonucleotide was synthesized: B-GLN, ACTGATCAAATTTATC, homologous to the 5’ end of apo B-100 mRNA at position 6679 (Davidson et al. 1988a). The oligonucleotide was radiolabeled by incubation with T4 polynucleotide kinase as previously described (Powell et al. 1987). Hybridization of Southern blots to 5’-GTGACGTTGACATCC (coding) and 5’-GTGACGTTGACATTT (non-coding) was denatured at 94 °C for 1 min, and extended at 72 °C for 5 min followed by cooling slowly to 42 °C in a buffer containing 100 mM NaCl, 10 mM Tris, pH 7-8, and 1 mM EDTA. Following ethanol precipitation, the samples were resuspended in 50 mM Tris, pH 8-3, 3 mM MgCl2, 10 mM dTTP, 50 µM each dATP, dCTP, and dTTP, and 250 µM dideoxy GTP. Extension was conducted for 2 h at 42 °C using 10 U MMLV reverse transcriptase. Following ethanol precipitation, the extension products were resolved by 8-5% polyacrylamide-urea gel electrophoresis and detected by autoradiography at –80 °C. Based on the nucleotide sequence of rat apo B mRNA (Davidson et al. 1988a), the predicted products are 33 (CAA at nucleotide 6666) or 38 (UAA at nucleotide 6666), next C at nucleotide 6661) bases corresponding to abundance of apo B-100 and B-48 mRNA respectively. The intensity of the signals from unedited, apo B-100 and edited apo B-48 transcripts were determined by video-assisted densitometry or by using a phosphoimager (Molecular Dynamics, Uppsala, Sweden). The percentage of apo B-100 (CAA, unedited) and of B-48 (UAA, edited) was calculated by the signal from apo B-100 or B-48 by the sum of both signals.

**Hepatocellular extracts**

The S-100 fraction of hepatocytes was prepared according to the methods described previously (Dignam et al. 1983). The preparation of each sample required the pooling of livers of two rats from the control or treated group. Ammonium sulfate precipitation was used to concentrate apo B-100 editing activity in the S-100 fraction (Kristina et al. 1990).

**In vitro editing assay**

The relative hepatic apo B-100 editing activity from control and treated rats was assayed using methods described previously (Kristina et al. 1990, Sowden et al. 1996). Each reaction contained 50 µg hepatocellular protein from control or treated animals incubated for 3 h at 30 °C with 10 ng apo B-100 RNA fragment, 6512–6786. The resulting products were analyzed using primer extension as described above and the intensity of the signals assessed using a phosphoimager or video-densitometry. The 6512–6786 segment of apo B-100 RNA was obtained by inserting the product of a PCR primed with PCR1 plus PCR3 using rat genomic DNA as template into pGEM-T (Promega). This construct was digested with SacI.
which cleaves the insert at the 3′ end and T7 RNA polymerase (Pharmacia) was used as per the manufacturer’s directions to produce the RNA. In a similar fashion, apo B-48 RNA 6512–6786 was obtained by T7 polymerase transcription from pGEM-T containing the fragment obtained by RT-PCR using total hepatic RNA as the template. Both the apo B-100 and -48 clones were verified by sequencing.

Cell culture
Human hepatoma Hep G2 cells were grown under previously described conditions (Murao et al. 1998). Cells grown to 70% confluence were exposed to 10^{-9} or 10^{-7}M CGS 23425 for 48 h. RNA was isolated from the cells as described above (Barrera-Hernandez et al. 1996). The content of apo AI mRNA was assayed by Northern-blot (Murao et al. 1998) and apo B mRNA levels were measured as described above.

RESULTS

CGS 23425 decreases postprandial levels of triglyceride in rats
To examine the effect of a thyromimetic on postprandial triglyceride clearance, rats were gavaged with water or an aqueous solution of CGS 23425. Results (Fig. 1) showed that serum triglyceride clearance was significantly hastened by CGS 23425. Triglyceride levels returned to baseline values within 8 h in rats treated with CGS 23425, whereas the same parameter in control rats was still elevated at 24 h after fat ingestion. Furthermore, the excursion above the baseline triglyceride value in treated animals was less than that in the controls.

CGS 23425 decreases levels of apo B-100 protein
Since triglyceride clearance was significantly accelerated by CGS 23425 treatment, we wondered whether this drug affected the levels of apo B in a hyperlipidemic rat model. Therefore, Western blot analysis was used to measure abundance of apo B-100 and apo B-48 in the plasma of rats treated with or without 30 µg/kg/day CGS 23425 for 0, 6 and 9 consecutive days. Results (Fig. 2) showed that levels of apo B-100 decreased by 80% and 40% following 6 and 9 days of treatment, respectively, compared with control. However, apo B-48 levels remained the same in all groups. As expected, in hyperthyroid rats the apo B-100 levels were barely detectable and apo B-48 levels did not change significantly (Fig. 2).

CGS 23425 lowers apo B-100 protein in hypercholesterolemic rats
The above findings showed that CGS 23425 decreased levels of apo B-100 at a fixed dose. To define the effective range of this drug, we used Western-blot analysis to measure the levels of apo B-100 and B-48 in fat-fed rats treated with various doses of CGS 23425. Results (Fig. 3) showed a dose-dependent decrease in apo B-100 protein. The minimal effective dose was 10 µg/kg and the maximum dose tested was 300 µg/kg leading to reductions of 50% and 83% respectively. However, apo B-48 levels remained essentially the same at all doses.

CGS 23425 decreases hepatic apo B-100 mRNA
The marked reduction in serum apo B-100 protein prompted us to examine whether the effect of CGS 23425 extends to apo B-100 mRNA levels in the liver of control and treated hyperlipidemic rats. In order to use RT-PCR as a semi-quantitative measure of apo B mRNA, the cDNA product from reactions after 22, 24, 26 and 28 cycles was examined by ethidium bromide staining. Results
(Fig. 4A) show a linear increase in abundance of the product at each cycle. A comparison of the slopes derived from the increase of total apo B cDNA showed a slight decrease in the treated versus control sample. The decision to use 25 cycles of PCR to assess abundance of apo B mRNA arises from the findings in Fig. 4A.

Consistent with the observation in Fig. 4A, RT-PCR amplification of hepatic RNA from 4 rats prior to and following 9 days of treatment with CGS 23425 revealed a slight decrease in total apo B cDNA in treated versus control animals. This decrease is evident by comparing the intensity of the total apo B cDNA in control versus CGS 23425-treated rats (Fig. 4B, compare lanes 1–4 with 5–8 in the panel designated total apo B). In contrast, there is no difference in abundance of β-actin mRNA between control and treated rats (Fig. 4B, middle panel). Since total apo B cDNA is a single band, it reflects abundance of both apo B-100 and B-48 mRNAs. To determine whether there was a selective decrease in apo B-100 mRNA, we further analyzed the RT-PCR product using Southern-blot hybridization with a radiolabeled probe that is specific for apo B-100 cDNA. The results (Fig. 4B, lower panel) showed apo B-100 cDNA from the liver of treated rats was decreased by 37% compared with that in the controls.

Since RT-PCR is semi-quantitative, we used direct RNA primer extension to measure abundance of apo B-100 mRNA. Apo B-100 mRNA was significantly decreased in CGS 23425-treated versus...
control rats (Fig 4C, compare B-100 signal in lane 1 with lane 2). The relative abundance of hepatic apo B-100 mRNA was compared with that of the B-48 isoform. In 4 control rats apo B-100 was 48% ± 5% of total apo B mRNA, whereas in 4 CGS 23425-treated rats apo B-100 was only 21% ± 5%. The 27% reduction in abundance of apo B-100 mRNA in treated animals was similar to that observed using RT-PCR (see above). In a hyperthyroid rat (Fig. 4C, lane 4), the level of apo B-100 mRNA decreased to 15%. Conversely, a hypothyroid rat with undetectable levels of L-T3, had very high levels of apo B-100 that accounted for 90% of the total apo B mRNA (Fig. 4C, lane 3). Together these studies show that like L-T3, CGS 23425 decreases the hepatic levels of apo B-100 mRNA in rats.

CGS 23425 increases apo B-100 mRNA editing activity

To determine whether changes in hepatic apo B-100 editing activity may account for the higher levels of apo B-48 mRNA in treated rats, the S-100 fraction from hepatocytes was assayed in vitro for apo B-100 editing activity (Kristina et al. 1990, Sowden et al. 1996). Each reaction contained the substrate, apo B-100 RNA fragment 6512–6786, incubated with S-100 extract, concentrated with ammonium sulfate, from the liver of control or treated rats. The product of the reaction was assayed for its content of unedited apo B-100 (CAA) and edited B-48 (UAA) RNA using primer extension. Results (Fig. 5A, compare apo B-48 signal in control and treated lanes (lanes 3 and 4)) showed that the edited, UAA isoform was higher in the extract from treated rats compared with control. Separate repetitions of this reaction using extracts from 3 control and 3 treated animals revealed a relative abundance of the edited UAA product that was 36% ± 6% as compared with 18% ± 4% in treated rats. These studies show that apo B-100 editing activity in liver extract is twofold higher in treated rats compared with that in control rats.

Absence of apobec-1 blocks effect of CGS 23425

If the actions of CGS 23425 require apo B-100 editing activity, then treating cells that lack apobec-1 with the agent should not affect the ratio of apo B-100 to B-48 mRNA. Since human liver does not edit apo B-100 mRNA, we treated human hepatoma Hep G2 cells with CGS 23425 because they have no detectable apobec-1 activity (Lau et al. 1994). Abundance of apo B-100 and B-48 mRNA in control and treated cells was measured using primer extension. Results (Fig. 5B) showed that apo B-100 mRNA levels were the same in both sets of cells. To show that the thyromimetic was indeed active...
Taylor et al. 1997), we measured the levels of apo AI mRNA in cells treated with $10^{-9}$ and $10^{-7}$ M CGS 23425 and detected increased values of $1.4 \pm 0.2$ and $1.9 \pm 0.3$, respectively, compared with control (Fig. 5C). These studies further support the idea that, like $\mathrm{L-T}_{3}$, the effects of CGS 23425 on apo B-100 mRNA require apobec-1.

**Discussion**

In this report we have examined the actions of a novel thyromimetic, CGS 23425, on expression of apo B-100 in fat-fed rats. This compound is of clinical interest for the following reasons: (i) it raises the levels of apo AI expression, (ii) at the same time...
it lowers levels of cholesterol and (iii) it has no cardiotoxicity at doses that exceed therapeutic concentrations by 4000-fold (Taylor et al. 1997). Although the beneficial actions of this drug on apo AI and cholesterol metabolism have been examined, whether it had any effect on other parameters of fat metabolism was unknown. Therefore, we initially tested the actions of CGS 23425 on lipid metabolism by measuring postprandial triglyceride levels in vivo (Fig. 1). Results showed the ability of CGS 23425 to inhibit the marked rise in triglyceride levels following an oral fat load. This observation prompted us to ask whether actions of the compound may affect expression of apo B because of its connection with triglyceride metabolism. However, we have not explored the potential effects of CGS 23425 on apo CII, the major pathway for the clearance of post-prandial

**FIGURE 5.** CGS 23425 increases apo B-100 editing activity in rat hepatic S-100 extract but has no effect on human Hep G2 cells. (A) Results of primer extension analysis of RNA product arising from apo B-100 mRNA editing activity in the S-100 fraction, precipitated with ammonium sulfate, from the livers of control and CGS 23425-treated rats. The contents of each lane are (1) apo B-48 mRNA fragment 6512–6786; (2) apo B-100 mRNA fragment 6512–6786; (3) apo B-100 mRNA fragment 6512–6786 treated with extract from a control and (4) CGS 23425-treated rat. (B) Primer extension analysis of apo B-100 mRNA in samples from Hep G2 cells treated without (lanes 1 and 2, control) and with (lanes 3 and 4) 10^{-7} M CGS 23425. (C) Graph of the relative amounts of apo AI mRNA in Hep G2 cells treated without (control) and with 10^{-9} or 10^{-7} M CGS 23425. Equivalent amounts of total RNA loaded into each lane were assessed by ethidium bromide staining prior to transfer and hybridization with a radiolabeled apo AI cDNA (data not shown). Each bar represents the mean ± s.e.m. of n=4. *P<0.05: significantly different from the control (ANOVA).
triglycerides; further studies exploring this possibility are needed.

Therefore, we examined the effects of CGS 23425 on plasma apo B levels in fat-fed hypercholesterolemic rats treated with a daily single dose of the drug for 6 and 9 days (Fig. 2). The results of these studies showed that CGS 23425 lowered the levels of apo B-100 protein, but had little or no effect on abundance of apo B-48. Not surprisingly, this action of the thyromimetic paralleled those of L-T₃ (Davidson et al. 1988a,b). The levels of apo B-100 protein were barely detectable in hyperthyroid rats.

A variety of mechanisms may underlie the ability of CGS 23425 to lower levels of apo B-100 protein. However, we chose to study its effects on apo B-100 mRNA because this drug is a thyromimetic and its parent hormone, L-T₃, is known to enhance the editing of apo B-100 mRNA. To examine this effect, the levels of hepatic apo B-100 mRNA in the liver of control and treated rats were measured using RT-PCR and primer extension analysis. Results (Figs 3 and 4) showed significant decreases in the abundance of apo B-100 mRNA following treatment with CGS 23425. As expected, in various thyroid states levels of apo B-100 mRNA correlated inversely with abundance of the hormone. For example, in hypothyroid rats the apo B-100 mRNA was high relative to that of apo B-48 because of low apo B-100 editing activity. This comparison was reversed in hyperthyroid animals. Together, these studies show that, like L-T₃, the liver of rats treated with CGS 23425 have a lower content of apo B-100 protein and mRNA.

Although the preceding studies show that CGS 23425 decreases levels of apo B-100, the mechanism for its action(s) remained unknown. CGS 23425 is a thyromimetic and since L-T₃, is known to enhance the editing of apo B-100 mRNA to apo B-48, the same pathway may mediate the conversion of apo B-100 mRNA to its B-48 isoform. This comparison was also reversed in hyperthyroid animals. Together, these studies show that, like L-T₃, the liver of rats treated with CGS 23425 have a lower content of apo B-100 protein and mRNA.

In conclusion, the present findings indicate that the thyromimetic, CGS 23425, lowers the hepatic levels of apo B-100 protein and mRNA in...
hyperlipidemic rats. This decrease appears to be mediated, in part, by enhancing the editing of the apo B-100 mRNA. The mechanism for this action of CGS 23425 is different from its nuclear effects that increase apo AI gene transcription. An added effect of the compound is that it decreases the levels of postprandial triglyceride. These studies define the actions of CGS 23425 in rats.

ACKNOWLEDGEMENTS

Funding for this project was provided by the Medical Research Council of Canada (MRC) and the Heart and Stroke Foundation of Canada to N C W W. G M H is the recipient of a post-doctoral fellowship from the Alberta Heritage Foundation for Medical Research. N C W W is the recipient of Scientist awards from the Alberta Heritage Foundation for Medical Research and the MRC. Funding for this project was provided by the Albert Heritage Foundation for Medical Research. N C W W. G M H is the recipient of a post-doctoral fellowship from the Heart and Stroke Foundation of Canada to N C W W.

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REVISED MANUSCRIPT RECEIVED 24 July 2000