Apolipoprotein A-I_{Milano} and Apolipoprotein A-I_{Paris} Exhibit an Antioxidant Activity Distinct from That of Wild-Type Apolipoprotein A-I[†]

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ABSTRACT: Apolipoprotein A-I_{Milano} (apoA-I_{Milano}) and apoA-I_{Paris} are rare cysteine variants of apoA-I that produce a HDL deficiency in the absence of cardiovascular disease in humans. This paradox provides the basis for the hypothesis that the cysteine variants possess a beneficial activity not associated with wild-type apoA-I (apoA-I_{WT}). In this study, a unique antioxidant activity of apoA-I_{Milano} and apoA-I_{Paris} is described. ApoA-I_{Milano} was twice as effective as apoA-I_{Paris} in preventing lipoxygenase-mediated oxidation of phospholipids, whereas apoA-I_{WT} was poorly active. Antioxidant activity was observed using the monomeric form of the variants and was equally effective before and after initiation of oxidative events. ApoA-I_{Milano} protected phospholipid from reactive oxygen species (ROS) generated via xanthine/xanthine oxidase (X/Xo) but failed to inhibit X/Xo-induced reduction of cytochrome *c*. These results indicate that apoA-I_{Milano} was unable to directly quench ROS in the aqueous phase. There were no differences between lipid-free apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT} in mediating the efflux of cholesterol from macrophages, indicating that the cysteine variants interacted normally with the ABCA1 efflux pathway. The results indicate that incorporation of a free thiol within an amphipathic α helix of apoA-I confers an antioxidant activity distinct from that of apoA-I_{WT}. These studies are the first to relate gain of function to rare cysteine mutations in the apoA-I primary sequence.

Cardiovascular disease (CVD)[1] is the number one cause of death in Western societies, and its prevalence is increasing worldwide. One of the strongest predictors of risk is the plasma concentration of high-density lipoprotein (HDL), which exhibits an inverse relationship (*1*, *2*). Despite the strong epidemiological data relating increased plasma HDL to protection against CVD, a number of rare inheritable traits have been described which result in low plasma HDL concentrations but no increase in CVD. These inheritable traits are, in part, attributed to mutations in apolipoprotein A-I, the major protein component of HDL (*3*).

Apolipoprotein A-I_{Milano} and apoA-I_{Paris} are examples of natural variants of apoA-I that manifest HDL deficiencies, but there is no apparent CVD in affected subjects (*4*−*6*). Indeed, a recent clinical study showed that carriers of apoA-I_{Milano} exhibited normal intimal thickness of carotid arteries compared to age- and sex-matched controls, whereas hypo-α-lipoproteinemic individuals showed intimal thickening as judged by B-mode ultrasound (*7*). Studies utilizing mice and rabbits support clinical studies by demonstrating that injection of recombinant apoA-I_{Milano} protects against atherosclerosis (*8*−*10*). However, the mechanism(s) by which apoA-I_{Milano} exerts its antiatherogenic effects is/are not completely understood.

All known human carriers of apoA-I_{Milano} and apoA-I_{Paris} are heterozygous for R173C and R151C mutations in the apoA-I primary sequence, respectively (*4*, *6*). The introduction of a cysteine residue in a normally cysteine-free apolipoprotein allows for the formation of homodimers and heterodimers with apoA-II. Dimerization of the cysteine variants inhibits HDL maturation via mechanisms related, in part, to impaired activation of lecithin:cholesterol acyltransferase, the enzyme that catalyzes cholesterol esterification on HDL (*11*−*13*). ApoA-I_{Milano} and apoA-I_{Paris} are rapidly cleared from the plasma compartment in humans, thus contributing to the HDL deficiency in vivo (*14*, *15*). However, the fractional catabolic rate of apoA-I_{Paris} appears to be different from that of apoA-I_{Milano}, suggesting that the two cysteine variants may differ in their metabolic behavior. Human carriers of apoA-I_{Milano} and apoA-I_{Paris} also exhibit mild hypertriglyceridemia in addition to the HDL deficiency (*6*, *16*).

High-density lipoproteins (HDL) from carriers of apoA-I_{Milano} consist of heterogeneous mixtures of small, dense HDL_3-sized particles as determined by rate zonal ultracentrifugation, whereas control subjects (noncarriers) possess mixtures of HDL_3 and large, less dense HDL_2 subpopulations (*17*). The monomeric form of apoA-I_{Milano} can be found on the surface of HDL_3 ranging from 30% to 40% of the apoA-I mass depending on the specific HDL_3 fraction analyzed (*17*).

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[1] Abbreviations: CVD, cardiovascular disease; apoA-I, apolipoprotein A-I; X/Xo, xanthine/xanthine oxidase; GSH, reduced glutathione; ROS, reactive oxygen species; SOD, superoxide dismutase; ABCA1, ATP-binding cassette transporter.

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The amount of monomeric apoA-I_{Paris} relative to its dimeric form on specific HDL subpopulations is currently unknown, but approximately 10% of the total plasma pool of apoA-I consists of monomers of apoA-I_{Paris} in vivo (*15*).

The paradox of abnormal lipoprotein metabolism and protection from CVD has led to the suggestion that the cysteine mutation in the lipid-binding domain of apoA-I may impart a gain of function protecting against atherosclerosis. As thiol groups in proteins are strong nucleophiles often participating in electron-transfer reactions, we hypothesized that the monomeric forms of apoA-I_{Milano} and apoA-I_{Paris}, which contain a free thiol, may possess an antioxidant activity distinct from that of apoA-I_{WT}. The results of our studies indicate that apoA-I_{Milano} and, to a lesser extent, apoA-I_{Paris} were potent inhibitors of lipid peroxidation, protecting phospholipid surfaces from lipophilic as well as water-soluble free radical initiators, whereas apoA-I_{WT} was a relatively poor inhibitor of oxidative events.

MATERIALS AND METHODS

Materials. The following reagents were purchased from Sigma (St. Louis, MO): soybean lipoxygenase, superoxide dismutase, xanthine, and cytochrome *c*. Xanthine oxidase was purchased from Roche Diagnostics (Mannheim, Germany). The phospholipid 1-palmitoyl-2-linoleoylphosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). [^{14}C]Cholesterol was purchased from Amersham Pharmacia (Buckinghamshire, England).

Recombinant Apolipoproteins. ApoA-I_{Milano} (R173C) and apoA-I_{Paris} (R151C) were prepared by primer-directed mutagenesis (*18*). The mutations were verified by dideoxy flourescent sequencing of cDNAs. Mutagenized cDNAs as well as a cDNA encoding apoA-I_{WT} were subcloned into a pET-20b bacterial expression vector. The plasmids were transformed into *Escherichia coli* strain BL21(DE-3) pLysS and expressed proteins purified via His-Trap chelating columns (Pharmacia Inc.) as described (*18*). During the isolation process, expressed proteins were maintained in denaturant (3 M guanidine hydrochloride in 20 mM phosphate and 0.5 M NaCl, pH = 7.4) and washed in 20 mM phosphate and 0.5 M NaCl (pH = 7.4) buffer just prior to column elution (*18*). This helps to ensure that a high proportion of the cysteine variants are bound to the His-Trap columns in monomeric form, and the proteins assume a native conformation while attached to the column in the presence of physiological buffers. This manner of isolation reduces the percentage of disulfide-linked dimers. Three separate batches of each of the recombinant apolipoproteins were used throughout these studies. In all cases, the cysteine variants were predominantly monomeric (90 ± 5%). The small variation in the distribution of monomers and dimers was observed between different batches of recombinant apolipoproteins. Both cysteine variants were prepared simultaneously to ensure that the ratio of monomers to dimers was essentially identical for side-by-side comparisons as shown in Table 1 and the figures. SDS−PAGE analysis of the isolated proteins revealed differences in the electrophoretic migration between the dimeric forms of apoA-I_{Milano} and apoA-I_{Paris} (Figure 1). The difference in apparent molecular weight of dimeric apoA-I_{Paris}, compared to dimers of apoA-I_{Milano}, is probably related to the differences in the position of the cysteine substitutions in the apoA-I primary sequence. The cysteine substitution in apoA-I_{Paris} is exactly 22 amino acids away from the cysteine residue of apoA-I_{Milano}. This produces a shift in the alignment of disulfide-linked polypeptide chains causing the apoA-I_{Paris} homodimer to form a more elongated structure, relative to apoA-I_{Milano} dimers (*19*), which likely alters the hydrodynamic behavior of the apoA-I_{Paris} variant as judged by a shift in apparent molecular weight.

Assessment of Antioxidant Activity. The oxidation system consisted of a micelle substrate composed of 1-palmitoyl-2-linoleoylphosphatidycholine (3 mM) dispersed in borate (pH = 9.0)/saline−EDTA (2.7 mM) and deoxycholate (6 mM) as described (*20, 21*). Phospholipid micelles were used throughout most of these studies to optimize rates of lipid peroxidation catalyzed by specific enzymes. This permitted us to quantify initial rates reliably and in reproducible fashion. Soybean lipoxygenase (5 units/μL) and xanthine (0.2 mM)/xanthine oxidase (20 units/mL) were used to initiate lipid peroxidation following the addition of recombinant apolipoproteins to the phospholipid micelles. Increases in conjugated dienes (lipid peroxidation) were monitored by ultraviolet absorption spectroscopy (234 nm) at 25 °C. The mass of phospholipid hydroperoxides was calculated using the molar absorptivity coefficient (ϵ = 29500 L cm^{-1} mol^{-1}) of conjugated dienes. Initial rates (Table 1) of lipoxygenase-catalyzed lipid peroxidation were quantified from the linear portion of the oxidation curves as shown in Figure 2. In some experiments, the reduction of cytochrome *c* was monitored (550 nm) in response to superoxide anion production by xanthine/xanthine oxidase (*22*). This system was used to test the ability of apoA-I_{Milano} to quench reactive oxygen species (ROS) in a completely phospholipid-free system.

Cellular Cholesterol Efflux Studies. J774 mouse macrophages were used as cholesterol donors and lipid-free recombinant apolipoproteins as cholesterol acceptors. This cell line was chosen because it possesses an active apolipoprotein-mediated efflux pathway involving the ATP-binding cassette transporter ABCA1 that can be upregulated by the cAMP analogue 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (*23*). The capacity of apoA-I_{Milano} and apoA-I_{Paris} compared to apoA-I_{WT} to promote cellular cholesterol efflux was evaluated using unstimulated and stimulated cells. Briefly, 1×10^5 cells/mL were seeded into 24-well culture plates and labeled (48 h) with 1 μCi/mL [^{3}H]cholesterol dispersed in RMPI 1640 containing 1% FBS. After the labeling, cellular [^{3}H]cholesterol was quantified by 2-propanol extraction followed by liquid scintillation counting. A parallel set of confluent cultures was equilibrated (2 h) with RPMI containing 0.2% BSA, followed by extensive rinsing with serum-free RPMI. Lipid-free apoA-I_{WT}, apoA-I_{Milano}, and apoA-I_{Paris} were applied (25 μg/mL) to cells in serum-free RPMI (±cAMP analogue, 0.3 mM). At specified times, aliquots of the medium were taken, and cellular debris was removed by centrifugation (1000*g*, 10 min). Radioactivity in the medium supernatant was normalized to initial cell ^{3}H, and efflux was expressed as percentages.

Other Methods. Protein was measured by the method of Markwell (*24*) and SDS−PAGE performed as described by Laemmeli (*25*). Statistical analyses were performed using Student's unpaired *t*-test, and $p < 0.05$ was considered the cutoff for significance.

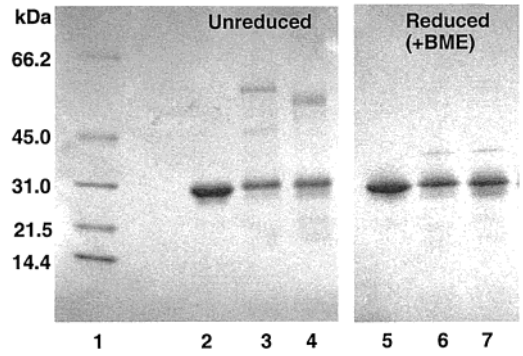

FIGURE 1: SDS−PAGE showing monomeric and dimeric forms of recombinant apoA-I_{Milano} and apoA-I_{Paris}. Lane 1 shows molecular weight standards; lane 2, recombinant apoA-I_{WT}; lane 3, apoA-I_{Paris}; lane 4, apoA-I_{Milano}; and lanes 5−7, apoA-I_{WT}, apoA-I_{Paris}, and apoA-I_{Milano} reduced with β-mercaptoethanol. Protein loads were 3 μg per well. Gels were stained with Coomassie R250.

RESULTS

The SDS−polyacrylamide gel in Figure 1 shows the monomeric and dimeric forms of recombinant apoA-I_{Milano} (R173C) and apoA-I_{Paris} (R151C). The bacterial expression system and isolation method yielded mostly (>90%) monomeric apolipoproteins. A small percentage (<10%) of each variant was in dimeric form. No differences were observed between apoA-I_{Milano} and apoA-I_{Paris} with regard to the distribution of monomers and dimers, and each of the recombinant apolipoproteins was greater than 95% pure as judged by Coomassie staining. For these reasons, the isolated proteins shown in lanes 2−4 were used for oxidation studies.

The antioxidant activity of recombinant apolipoproteins is shown in Figure 2. A micellar substrate composed of phospholipid and deoxycholate was exposed to lipoxygenase with or without addition of apolipoproteins. Panels A, B, and C (Figure 2) depict the dependence of antioxidant activity on the concentration of apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT}, respectively. ApoA-I_{Milano} was nearly twice as effective as apoA-I_{Paris} in preventing lipoxygenase-mediated oxidation of phospholipid (50% versus 27% protection at 50 μg of protein/mL at 8 min, respectively). ApoA-I_{WT} was a weak inhibitor at high doses (Figure 2, panel C). Unlike apoA-I_{WT}, protection against oxidation was evident at low doses of apoA-I_{Milano} (25 μg/mL) and at the initial stages (<1 min) of lipid peroxidation. Detailed time−course studies using high doses (i.e., 75 μg/mL) of apoA-I_{WT} (Figure 2, panel C) revealed a small degree of protection at the later times (>3 min) when lipid peroxides accumulated to high levels (>20 μM). Table 1 shows the initial rates of lipid peroxidation in the presence and absence of recombinant apolipoproteins. ApoA-I_{Milano} was found to inhibit the initiate rate of oxidation, whereas apoA-I_{Paris} behaved similar to apoA-I_{WT}, demonstrating no apparent impact on initial rates. Indeed, the antioxidant activity of apoA-I_{Paris}, compared to apoA-I_{WT}, was restricted to later times (>3 min) after the initial phase of lipid peroxide formation.

Table 1: Initial Rates of Lipoxygenase-Mediated Oxidation of Phospholipid and Protection Afforded by ApoA-I_{Milano}

apolipoprotein[a]	conjugated dienes[b] (nmol min^{-1} mL^{-1})	*P* value
no apolipoprotein ($n = 8$)	5.58 ± 0.74	
apoA-I_{WT} ($n = 5$)	5.10 ± 0.46	0.1642
apoA-I_{Paris} ($n = 3$)	4.80 ± 0.76	0.1959
apoA-I_{Milano} ($n = 5$)	2.87 ± 0.56	<0.0001

[a] The concentration of each of the recombinant apolipoproteins was 50 μg of protein/mL. [b] Means ± SD are shown. *P* values are compared to the "no apolipoprotein" controls and were calculated using Student's two-tailed test, adjusting for sample sizes.

Figure 3, panel A, shows that apoA-I_{Milano} was an effective antioxidant when added before and after initiation of lipid peroxidation with lipoxygenase. Antioxidant activity was specific to monomeric apoA-I_{Milano} as covalent dimers inhibited oxidation poorly like apoA-I_{WT} (i.e., absorbances at 234 nm after 10 min of lipoxygenase treatment were 1.14 and 1.08 for apoA-I_{Milano} dimers and apoA-I_{WT} each at 50 μg of protein/mL, respectively). A summary of several experiments using a single dose (50 μg/mL) of recombinant apolipoproteins is shown in Figure 3, panel B. Note that apoA-I_{Milano} was consistently more effective than apoA-I_{Paris} in preventing the oxidation of phospholipid. The water-soluble thiol compound, reduced glutathione (GSH, 0.1 mM), failed to inhibit lipid peroxidation. The lack of effect of GSH alone is probably related to the fact that this tripeptide is

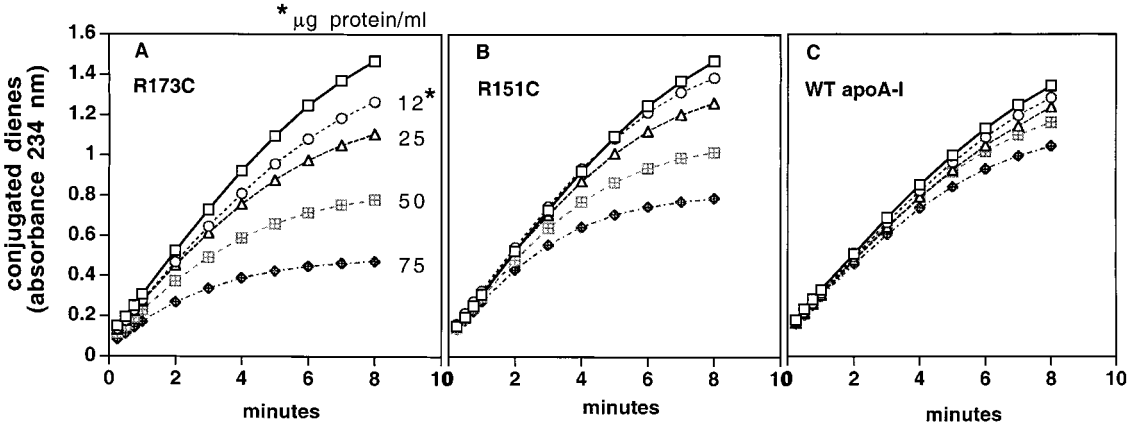

FIGURE 2: Antioxidant activity of recombinant apoA-I_{Milano} and apoA-I_{Paris}. Panels A, B, and C show the antioxidant activity of apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT}, respectively, on phospholipid micelles (Materials and Methods) exposed to soybean lipoxygenase (5 units/μL). Apolipoprotein concentrations were the same in each panel as shown for apoA-I_{Milano} (panel A). Open squares in all of the line graphs represent oxidation with no apolipoproteins. Lipid peroxidation was quantified by measuring conjugated dienes (i.e., absorbance at 234 nm). Representative data are shown from a total of at least three separate experiments. An asterisk indicates protein concentrations (μg/mL) and corresponding symbols.

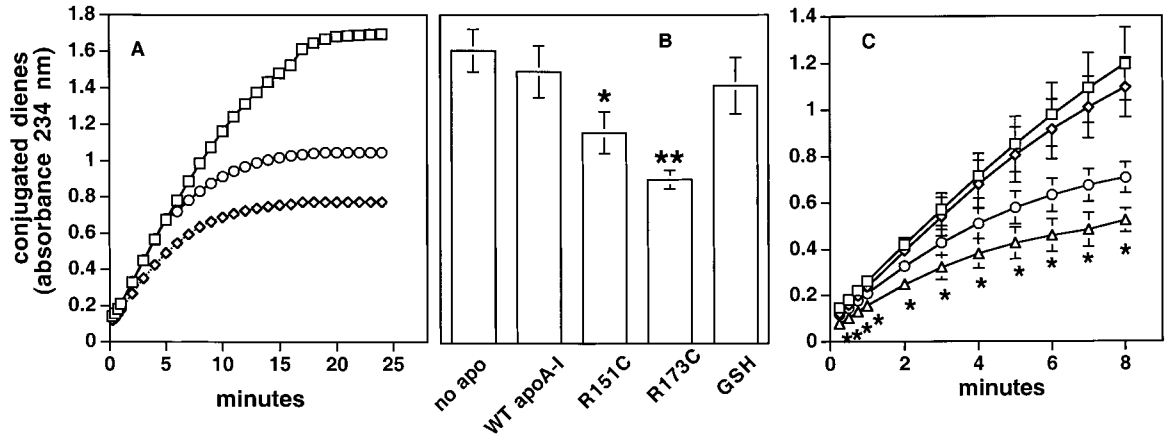

FIGURE 3: Characteristics of the antioxidant activity of apoA-I_{Milano}. Panel A shows that apoA-I_{Milano} (50 μg/mL) prevented oxidation of phospholipid before (diamonds) and after (circles) initiation with soybean lipoxygenase. Note that maximum levels of lipid peroxidation were achieved by 20 min of incubation in the absence of apoA-I_{Milano} (squares). Results are representative of two independent experiments. The bar graph (panel B) shows a summary of the full extent (24 min) of lipid peroxidation in the presence and absence of apolipoproteins (50 μg/mL). Values are means ± SD, $n = 3$ (*, $p < 0.05$ compared to apoA-I_{WT}, and **, $p < 0.05$ compared to apoA-I_{Paris}). Reduced glutathione (GSH, 0.1 mM) failed to inhibit lipid peroxidation. Panel C shows the interaction between GSH (0.1 mM) and apoA-I_{Milano}: squares, no additions; diamonds, GSH alone (0.1 mM); circles, apoA-I_{Milano} alone (50 μg of protein/mL); and triangles, apoA-I_{Milano} plus GSH. Values are means ± SD, $n = 3$ (*, $p < 0.02$ compared to apoA-I_{Milano} alone).

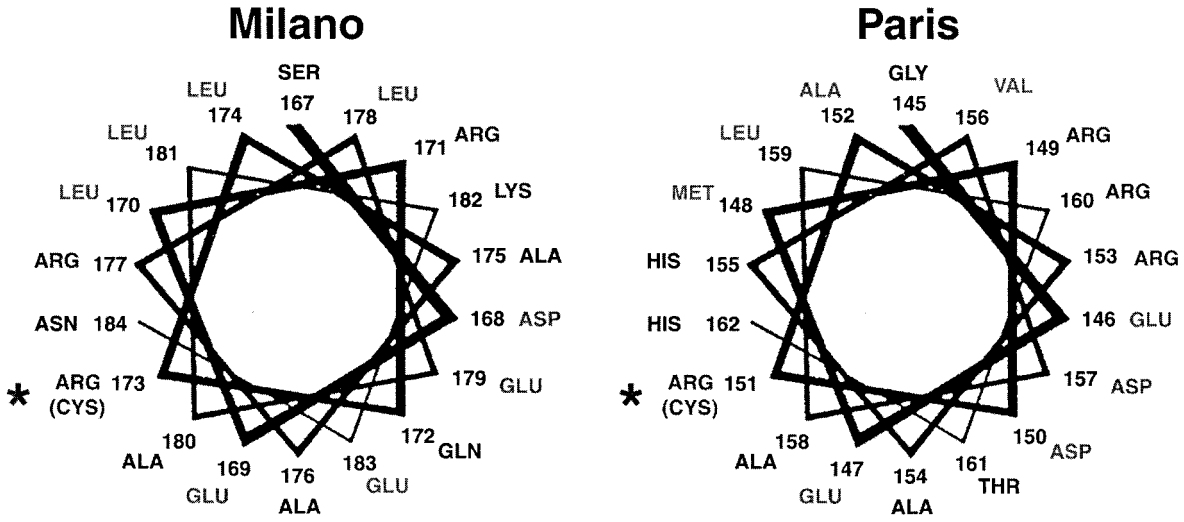

FIGURE 4: Helical wheel projections showing the position of R → C mutations in amphipathic α helices of apoA-I. Numbers reflect the position of amino acids in the apoA-I primary sequence. Note the similarities between apoA-I_{Milano} (R173C) and apoA-I_{Paris} (R151C). Both R → C mutations are located near the hydrophobic/hydrophilic interface of each helix. The substitution of cysteine for arginine disrupts salt bridges normally associated with Arg173−E169 and Arg151−E147 (*33*).

water soluble, whereas lipoxygenase must bind to the surface of the phospholipid micelle to catalyze lipid peroxide formation. This suggests that water-phase GSH does not interfere with the lipoxygenase reaction on phospholipid surfaces. However, the combination of apoA-I_{Milano} (50 μg/mL) and GSH (0.1 mM) acted synergistically, enhancing antioxidant activity compared to apoA-I_{Milano} alone (Figure 3, panel C). Analysis of the kinetic data revealed that GSH exerted its beneficial effects by enhancing (20 ± 2.4%, $n = 3$) the capacity of apoA-I_{Milano} to inhibit the initial rate of lipid peroxidation.

The helical wheel projections in Figure 4 indicate the position of R173C and R151C within amphipathic α helices of apoA-I. Both cysteine substitutions for arginine are located near the interface of the hydrophobic/hydrophilic surfaces of their respective amphipathic α helices. Because of this unique location of the cysteine residue near the hydrophobic/hydrophilic interface, it was hypothesized that apoA-I_{Milano} may protect phospholipid from water-soluble free radical initiators in addition to lipoxygenase-mediated mechanisms (Figures 2 and 3) which occur on phospholipid surfaces. Figure 5 shows the antioxidant activity of apoA-I_{Milano} using X/Xo to generate ROS in the aqueous phase of the reaction system. Two different terminal electron acceptors (i.e., phospholipid and cytochrome *c*) were used for these experiments. The results using phospholipid as a substrate for oxidation are shown in Figure 5, panel A. ApoA-I_{Milano} (50 μg/mL) inhibited X/Xo-induced lipid peroxidation by approximately 50%, consistent with the protection against lipoxygenase, whereas apoA-I_{WT} failed to protect phospholipid from ROS generated via X/Xo. These results indicate that the presence of a cysteine residue at the hydrophobic/hydrophilic interface of an amphipathic α helix protects phospholipid from water-soluble free radical initiators. However, apoA-I_{Milano} failed to inhibit X/Xo-mediated reduction of cytochrome *c* (Figure 5, panel B). These results indicate that apoA-I_{Milano} was unable to directly quench ROS (i.e., superoxide anion and hydroxyl radicals) in lipid-free

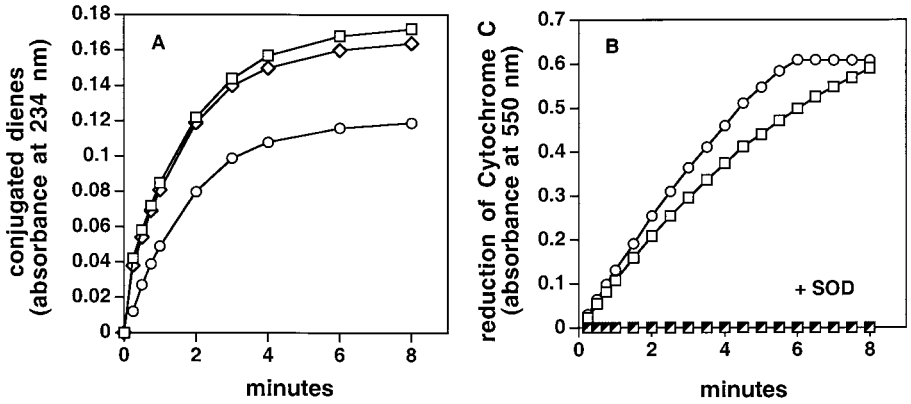

FIGURE 5: ApoA-I_{Milano} protects phospholipid from reactive oxygen species (ROS) generated via xanthine/xanthine oxidase (X/Xo). Panel A shows the oxidation of phospholipid using the micelle substrate as described in Figure 2 and xanthine (0.2 mM)/xanthine oxidase (20 units/mL) to initiate lipid peroxidation via the formation of ROS in the aqueous phase. Open squares represent oxidation in the absence of protective agent; diamonds, apoA-I_{WT} (50 μg/mL); and circles, apoA-I_{Milano} (50 μg/mL). Panel B shows the results from a phospholipid-free system based on the reduction of cytochrome *c*. ApoA-I_{Milano} (circles) failed to protect cytochrome *c* from ROS generated via X/Xo; open squares represent reduction of cytochrome *c* in the absence of apoA-I_{Milano}. Half-darkened squares represent a positive control using superoxide dismutase (SOD, 300 units/mL). Results in each panel are representative of two identical experiments.

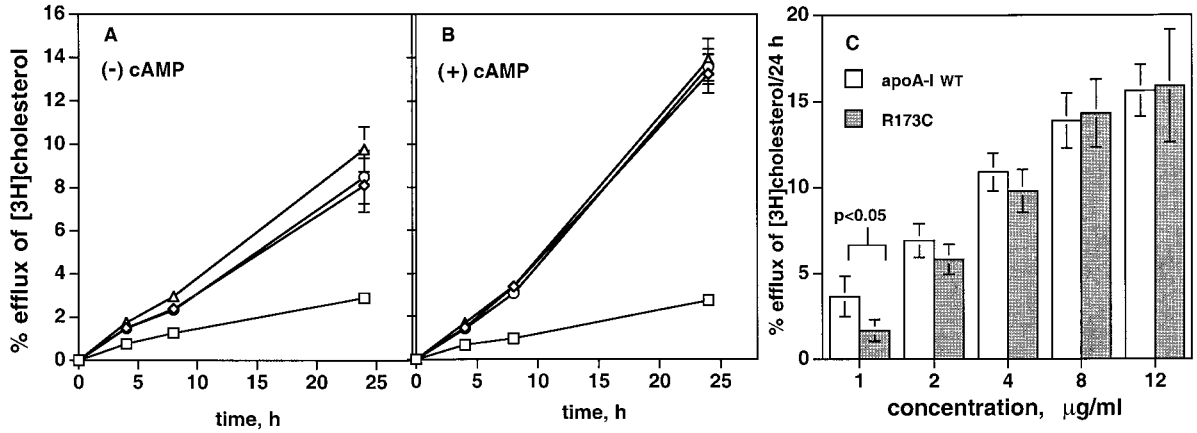

FIGURE 6: Capacity of lipid-free apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT} to promote cholesterol efflux. J774 mouse macrophages were radiolabeled with [^{3}H]cholesterol (1 μCi/mL per well) and exposed to apolipoprotein acceptors (25 μg/mL) as described in Materials and Methods. Panels A and B represent efflux from cells in the absence and presence of a cAMP analogue, respectively: squares, efflux to serum-free medium; circles, apoA-I_{WT}; diamonds, apoA-I_{Milano}; triangles, apoA-I_{Paris}. Panel C shows the dependence of cholesterol efflux from stimulated cells (24 h) on the concentration of apoA-I_{WT} and apoA-I_{Milano}. Results are expressed as a percentage of cellular [^{3}H]-cholesterol appearing in the medium. Values are means ± SD, $n = 3$.

form, suggesting that the antioxidant function of apoA-I_{Milano} was directed toward phospholipid. ApoA-I_{Paris} also protected phospholipid from X/Xo, but this variant was 50% less effective compared to apoA-I_{Milano}, consistent with the data presented in Figures 2 and 3 using lipoxygenase. Moreover, apoA-I_{Paris} failed to protect chytochrome *c* from ROS (data not shown).

One of the principal functions of apoA-I_{WT} is to promote the efflux of cholesterol from macrophages. This involves an obligatory interaction with the recently described ABCA1 gene product that is defective in Tangier's disease (*26*−*28*). Because this is an important process in preventing atheromatous lesions, the capacity of apoA-I_{Milano} and apoA-I_{Paris} to promote cellular cholesterol efflux using J774 mouse macrophages was compared to apoA-I_{WT}. No differences were observed between apoA-I_{WT} and the cysteine variants in mediating cholesterol efflux from unstimulated cells (Figure 6, panel A) consistent with recently published studies using apoA-I_{Milano} and apoA-I_{Paris} (*13*, *29*). Moreover, the capacity of apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT} to promote cholesterol efflux from stimulated J774 macrophages was the same for each of the recombinant apolipoproteins (Figure 6, panel B). Detailed dose−response curves shown in Figure 6 (panel C) indicate that apoA-I_{Milano} was somewhat less effective than apoA-I_{WT} in promoting cellular cholesterol efflux but only at very low doses (1 μg/mL). Similar results were also observed with apoA-I_{Paris} where the lowest dose examined (1 μg/mL) yielded reduced levels of cholesterol efflux compared to apoA-I_{WT} (data not shown). These results suggest that the putative antiatherogenic properties of apoA-I_{Milano} and apoA-I_{Paris} are unrelated to interactions with the ABCA1 transporter.

DISCUSSION

In this study, apoA-I_{Milano} (R173C) and apoA-I_{Paris} (R151C) were found to exhibit a potent antioxidant activity distinct from that of apoA-I_{WT}. Antioxidant activity of apoA-I_{Milano} and apoA-I_{Paris} was observed using the monomeric form of the variants. The monomeric forms of apoA-I_{Milano} and apoA-I_{Paris} possess a free sulfhydryl. As protein thiols are strong nucleophiles often associated with antioxidant activities, it is likely that the free cysteine residue in the variant apolipoproteins was responsible for the antioxidant activity not found with apoA-I_{WT}. The distinction between apoA-

I_{Milano} and apoA-I_{WT} is most clearly illustrated in Table 1 and the concentration dependence studies (Figure 2) where apoA-I_{Milano} was much more effective than apoA-I_{WT} in preventing lipoxygenase-mediated oxidation of phospholipid. ApoA-I_{Milano} protected against lipid peroxidation during the early stages of the reaction when lipid peroxide accumulation was minimal. Moreover, the concentrations of apoA-I_{Milano} used in these studies appear to be physiologically relevant as carriers possess roughly 50−150 μg/mL of apoA-I_{Milano} monomers in plasma (*15*). In contrast, the apparent antioxidant activity of apoA-I_{WT} was first evident at relatively high peroxide concentrations (approximately 20 μM) using high doses (75 μg/mL) of apolipoprotein. The latter is most likely attributed to the presence of specific methionine residues in apoA-I that have been shown to confer a peroxidase-like activity to apoA-I_{WT} (*30*−*32*).

It is unclear why apoA-I_{Milano} was twice as effective as apoA-I_{Paris} in protecting phospholipid from oxidation, but we suggest that this may be due to a contextual constraint governed by adjacent amphipathic α helices in the C-terminal lipid-binding domain of apoA-I. As noted in the helical wheel projections in Figure 4, the monomeric forms of apoA-I_{Milano} and apoA-I_{Paris} possess a free cysteine residue at the hydrophobic/hydrophilic interface of their respective amphipathic α helices. The presence of an uncharged cysteine residue disrupts putative salt bridges normally associated with Arg173−Glu169 and Arg151−Glu147, respectively (*33*). ApoA-I_{Milano} and apoA-I_{Paris} are similar with regard to the cysteine placement and salt bridge disruption, suggesting that sequences outside of these specific helices set the difference in antioxidant activity between the variants. This hypothesis is supported by preliminary results demonstrating that singular amphipathic α helices (18-mers) corresponding to helix 6 and helix 7, where R151C and R173C can be found, respectively, do not differ in antioxidant activity, exhibiting similar capacity to inhibit lipid peroxidation (data not shown).

The C-terminal lipid-binding domain of apoA-I consists of a series of helical repeats separated by proline residues. The amphipathic α helix (amino acids 167−184) containing R173C is flanked by two amphipathic α helices of relatively greater lipid-binding affinity. The lipid-binding affinity of the helical repeats alternate, but the two end helices of apoA-I exhibit the highest lipid-binding affinity (*34*). The relatively low lipid-binding affinity associated with helix 7, where R173C is located, may allow a high degree of movement of this particular helix on phospholipid surfaces, thus maximizing the frequency of collision between the free thiol at position 173 and reactive lipid peroxides. Increased flexibility of helix 7, which is located in the central region of the C-terminal lipid-binding domain, may be optimized in the presence of deoxycholate used in the preparation of the phospholipid micelles. Thus it remains to be determined whether the differences in antioxidant activity between apoA-I_{Milano} and apoA-I_{Paris} persist on native HDL particles and/or on the surface of reconstituted discoidal HDL complexes.

The antioxidant activity of apoA-I_{Milano} appeared to be directed toward phospholipid as the variant was unable to prevent superoxide anion mediated reduction of cytochrome *c* (Figure 5, panel B). This observation has important implications regarding the underlying mechanism by which apoA-I_{Milano} and apoA-I_{Paris} protect against oxidation. Xanthine/xanthine oxidase generates superoxide anion and hydroxyl radicals that are capable of initiating lipid peroxidation. The fact that apoA-I_{Milano} was unable to prevent X/Xo-mediated reduction of cytochrome *c* suggests that, in lipid-free form, apoA-I_{Milano} was unable to directly quench ROS in the water phase of this reaction system. These results do not rule out the possibility that an apolipoprotein conformational change and/or unfolding event on phospholipid surfaces is required for apoA-I_{Milano} to quench ROS. However, preliminary experiments conducted with a synthetic peptide of apoA-$I_{Milano,}$ which does not require unfolding, revealed that the peptides also failed to inhibit X/Xo-induced reduction of cytochrome *c* (data not shown), suggesting that exposure of the thiol to the water phase was not sufficient to quench ROS. Rather, the results suggest that apoA-I_{Milano} may act as a chain-breaking antioxidant inhibiting amplification events associated with the propagation of phospholipid hydroperoxides.

The oxidation of phospholipids is thought to occur via several mechanisms in the artery wall including specific lipoxygenase-mediated events and through the production of ROS by aortic cells (*35*, *36*). Common to these mechanisms is the formation of "seed" molecules (phospholipid peroxides) that can amplify oxidative events via free radical mediated abstraction of bisallelic hydrogen atoms from carbon−carbon double bonds of adjacent unsaturated fatty acyl chains producing conjugated dienes. This precedes the breakdown stage that results in the formation of oxidized phospholipids with shortened *sn*-2 chains. The cysteine variants of apoA-I could inhibit such an amplification event by quenching reactive lipid peroxides and/or reducing the lipid peroxide to its lipid alcohol form. By preventing amplification events of lipid peroxidation, apoA-I_{Milano} and apoA-I_{Paris} effectively inhibit the propagation of seed molecules. This renders phospholipids less susceptible to oxidation, thereby preventing the formation of fragmented phospholipid breakdown products that are proinflammatory mediators of atherogenesis.

ApoA-I_{WT} has been shown to extract lipid hydroperoxides from LDL and aortic endothelial cells (*37*, *38*). This function is probably related to the ability of apoA-I_{WT} to bind and transport lipid. Removal of seed molecules renders LDL resistant to subsequent metal ion dependent oxidation, thus preventing the formation of fragmented products that are proinflammatory. At present, we do not know whether apoA-I_{Milano} is more or less effective than apoA-I_{WT} in removing/transporting oxidized phospholipids from LDL or aortic cells. However, the cholesterol efflux studies indicate that the monomeric forms of apoA-I_{Milano} and apoA-I_{Paris} behave similarly to apoA-I_{WT} in mediating cellular cholesterol efflux via the ABCA1 transport pathway in macrophages. Apolipoprotein-mediated efflux requires the recruitment of membrane phospholipid. Thus, apoA-I_{Milano} is likely to behave similarly to apoA-I_{WT} in mediating the removal of oxidized phospholipids from cellular membranes and lipoprotein particles. Furthermore, the results presented here in conjunction with recent clinical data (*7*) on apoA-I_{Milano} carriers suggest that the antioxidative effects of apoA-I_{Milano} are more substantial than removal of seed molecules alone in inhibiting atherogenesis.

Studies of the cholesterol efflux properties of apoA-I_{Milano}, apoA-I_{Paris}, and apoA-I_{WT} provide additional insights into the potential antiatherogenic properties of the cysteine variants.

Lipid-free apolipoproteins can stimulate cholesterol efflux from cells via the recently described ABCA1 transport protein associated with cellular membranes (*26*−*28*). A number of studies, including our own, indicate that lipid-free apoA-I_{Milano} and apoA-I_{Paris} are just as effective as apoA-I_{WT} in mediating the efflux of cellular cholesterol via the ABCA1 efflux pathway (*28*, *29*). The only difference that we observed was at the very lowest concentration examined (i.e., 1 μg/mL) where the apoA-I cysteine variants were less effective than apoA-I_{WT} in mediating cholesterol efflux from J774 macrophages. Previous studies using Chinese hamster ovary cells transfected with the genes for either apoA-I_{Milano} or apoA-I_{WT} suggested that the former had reduced capacity to recruit membrane cholesterol for nascent HDL assembly (*39*). This is probably related to the relatively low levels of the secreted apolipoproteins appearing in the extracellular medium, which correspond to the low end of the dose−response curves shown in the present studies where apoA-I_{Milano} was less effective than apoA-I_{WT} in mediating cellular cholesterol efflux.

Cholesterol efflux from cells can also occur by an aqueous diffusion mechanism. By this mechanism, complexes composed of apoA-I and phospholipid acquire cellular cholesterol following its spontaneous desorption from the plasma membrane into the aqueous environment. This process is largely driven by a concentration gradient that favors net movement of membrane cholesterol to cholesterol-poor apolipoprotein/phospholipid acceptor particles. Calabresi et al. (*40*) observed that small complexes of apoA-I_{Milano} dimers and phospholipid were more efficient acceptors of cell-derived cholesterol compared to control complexes composed of apoA-I_{WT}. These investigators suggested that the enhanced efficiency of small apoA-I_{Milano} complexes may be related to specific structural changes in the C-terminal lipid-binding domain of the apoA-I_{Milano} dimers (*40*, *41*). Indeed, these investigators, using protease digestion techniques, provided evidence that a portion of the C-terminal lipid-binding domain of apoA-I_{Milano} homodimers may extend outward from the surface of the reconstituted HDL, thereby allowing the particles to associate with the plasma membrane of cells to facilitate cholesterol efflux.

In summary, the present study provides the first direct evidence that cysteine substitutions in the apoA-I primary sequence confer a gain of function, protecting phospholipid from free radical mediated lipid peroxidation. Since the initial stages of atherosclerosis are thought to involve the oxidation of phospholipid in the artery wall, it is likely that apoA-I_{Milano} and apoA-I_{Paris} may provide protection from atherosclerosis by inhibiting oxidative events to a greater extent compared to apoA-I_{WT}. Oxidized phospholipids with short *sn*-2 chains have been shown to stimulate the production of growth factors and cytokines that stimulate the expression of adhesion molecules on endothelial cells and modulate the binding of blood monocytes to the artery wall (*42*, *43*). The infiltration of monocytes into the artery wall and subsequent differentiation into macrophages and macrophage foam cells is a hallmark of developing atherosclerotic lesions. ApoA-I_{WT} is thought to inhibit some of these events by virtue of its capacity to remove phospholipid hydroperoxides and their breakdown products (i.e., fragmented phospholipids) from oxidized lipoproteins and aortic cells. However, the present studies suggest that apoA-I_{Milano} and apoA-I_{Paris} act at an earlier step, compared to apoA-I_{WT}, in the oxidation cascade, preventing phospholipid hydroperoxide formation/amplification. This suggests that the resistance to CVD associated with apoA-I_{Milano} and apoA-I_{Paris} in humans may be related, in part, to their ability to inhibit the formation of proinflammatory mediators of early atherogenesis. ApoA-I_{Milano} and apoA-I_{Paris} are likely to move in and out of the artery wall performing beneficial functions such as cellular cholesterol efflux via ABCA1 interactions. This coupled with the manifestation of potent antioxidant activity as the cysteine variants of apoA-I associate with phospholipid to form newly assembled HDL in the artery wall may confer a high degree of protection from atherogenic events. As such, therapeutic agents based on the monomeric forms of apoA-I_{Milano} and apoA-I_{Paris} hold promise as potent inhibitors of atherogenesis.

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