ORIGINAL ARTICLE

mRNA expression of genes involved in lipid efflux and matrix degradation in occlusive and ectatic atherosclerotic disease

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Background: Atherosclerotic plaque behaviour is influenced by intraplaque inflammation, matrix turnover, and the lipid core volume. Peroxisome proliferator activated receptor γ (PPARγ) modulates atherosclerosis by its anti-inflammatory and anti-protease activity. PPARγ promotes lipid efflux through the liver X receptor α (LXRα) and the ATP binding cassette transporter A1 (ABCA1). Matrix metalloproteinase 9 (MMP-9) and cyclooxygenase 2 (COX-2) are implicated in plaque instability.

Aims: To assess the expression of these genes in occlusive and ectatic atherosclerotic disease to determine the relation between genes involved in lipid efflux and matrix degradation.

Methods: Carotid endarterectomy specimens from 16 patients and aneurysm tissue from 16 patients undergoing abdominal aortic aneurysm repair were used. Inferior mesenteric arteries from colectomy specimens from 12 patients served as controls. Total RNA was extracted from pulverised tissue and reverse transcribed into cDNA. Quantitative real time polymerase chain reaction (PCR) was performed using fluorescently labelled probes for *ABCA1*, *LXRα*, *PPARγ*, *COX-2*, and *MMP-9*.

Results: *PPARγ* expression was significantly lower in both occlusive and ecstatic atherosclerotic disease ($p<0.001$), whereas *LXRα* and *ABCA1* expression was significantly increased ($p<0.01$). *MMP-9* expression was significantly increased in diseased tissues ($p<0.0001$), and values were highest in occlusive disease ($p<0.01$). The increases in *ABCA1* and *MMP-9* mRNA were significantly correlated in diseased tissues ($p<0.01$, $r=0.71$ and $r=0.78$). *COX-2* expression was increased in ectatic but low in occlusive disease ($p<0.01$).

Conclusion: This observational study suggests a role for therapeutic upregulation of PPARγ, which could potentially upregulate lipid efflux through ABCA1 and inhibit matrix degradation through inhibition of MMP-9.

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T he clinical progression of the atherosclerotic plaque is governed by the key processes of inflammation, lipid deposition, and matrix degradation. Increased uptake of modified low density lipoprotein (LDL) by monocytes/macrophages results in foam cell formation, the hallmark of an atheromatous plaque.[1] A large lipid core, formed from the extracellular accumulation of these foam cells,[2] is recognised as a risk factor for plaque rupture.[3] Increased activity of both proinflammatory cytokines and the matrix degrading matrix metalloproteinases, particularly in the region of the fibrous cap, can render the atherosclerotic plaque potentially unstable.[4]

> "A strategy that could combine both promotion of cholesterol efflux, and inhibition of inflammation and matrix degradation, would be of great benefit in treating or modifying plaque behaviour"

Ubiquitously expressed nuclear factor κB transcription pathways,[5] involved in the activation of cytokines such as cyclooxygenase 2 (COX-2),[6][7] have been implicated in plaque instability.[8] COX-2, mainly expressed in atherosclerotic lesions,[9] is involved in the release of matrix metalloproteinase 9 (MMP-9),[10] a matrix degrading protease, which has been reported to play a major role in atheromatous plaque rupture.[11] A strategy that could combine both promotion of cholesterol efflux, and inhibition of inflammation and matrix degradation, would be of great benefit in treating or modifying plaque behaviour.

Table 1 Demographic details of the patient and control groups

Characteristics	CEA group (n = 16)	Aneurysm group (n = 16)	Controls (n = 12)
Age (mean)	72	75	70
Sex			
Male	8	14	6
Female	8	2	6
Smoking	1	6	2
Diabetes	4	2	1
Hypertension	12	13	5
Symptomatic peripheral vascular disease	1	4	0
Drugs			
Aspirin	12	10	2
Clopidogrel	2	1	0
Warfarin	2	1	0
Statins	9	5	2

CEA, carotid endarterectomy.

One of the most exciting discoveries in recent years has been the peroxisome proliferator activated receptor γ (PPARγ), a nuclear receptor that is involved in the regulation

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Abbreviations: ABCA1, ATP binding cassette transporter A1; COX-2, cyclooxygenase 2; Ct, cycle threshold; LDL, low density lipoprotein; LXRα, liver X receptor α; MMP-9, matrix metalloproteinase 9; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator activated receptor γ

Table 2 Probes for the genes investigated

Genes	Probe (5'–3')
ABCA1	AACTTTAACAAATCCATTGTGGCTCGCCTGT
LXRα	CACTTCTAGGAGGCAGCCAC
PPARα	ACTTCAAGAGTACCAAAGTGCAATCAAAGTGGAG
COX-2	ATGATTGCCCGACTCCCTTGGGTGT
MMP-9	CCCAGCGAGAGACTCTACACCCGG

of both the inflammatory response and lipid homeostasis in the macrophage.[12] Nuclear receptors like PPARγ and the liver X receptor α (LXRα) are known to modulate atherogenesis at various stages from cell recruitment to lipid accumulation and the local inflammatory response.[13–16] PPARγ is known to suppress monocyte chemoattractant protein 1 expression,[17] inhibit adhesion molecules,[18 19] and facilitate cholesterol efflux.[20 21] It promotes cholesterol efflux via ATP binding cassette transporter A1 (ABCA1) (through the upregulation of LXRα) dependent[20] and distinctly ABCA1 independent pathways.[21] ABCA1, a membrane protein involved in cholesterol efflux, is highly expressed in macrophages[22] and plays a major role in high density lipoprotein metabolism.[23] It is also implicated in promoting macrophage engulfment of apoptotic cells.[24] Patients with ABCA1 deficiency states, such as Tangier disease,[25] have virtually no high density lipoprotein and are predisposed to premature atherosclerosis.[26]

PPARγ and LXRα are reported to suppress the synthesis of COX-2 and MMP-9 through their inhibitory effect on nuclear factor κB pathways.[13 14 27] It is interesting to note that PPAR agonists can effectively increase ABCA1 expression in addition to the suppression of proinflammatory pathways, including COX-2 and MMP-9.[16 28] All of these functions are pivotal in stabilising a potentially unstable atherosclerotic plaque. PPARγ has the potential to exert pleiotropic effects on the plaque by upregulation of lipid efflux and downregulation of genes governing inflammation. We aimed to evaluate the expression of genes involved in this lipid efflux pathway, and to assess its association with COX-2 and MMP-9 in occlusive and ectatic atherosclerotic disease.

METHODS

Patients and specimens

Sixteen carotid plaques were collected from patients with internal carotid artery stenoses of > 70% undergoing carotid endarterectomy. Fourteen patients were diagnosed with symptomatic carotid disease with a history of transient ischaemic attacks, strokes, or amaurosis fugax, whereas two were asymptomatic. Aneurysm wall specimens were collected from 16 patients undergoing elective abdominal aortic aneurysm repair. Twelve macroscopically normal inferior mesenteric arteries dissected from colectomy specimens of subjects undergoing elective surgery served as controls. These patients were phenotypically free of symptomatic atherosclerotic disease by history, examination, and a normal electrocardiography tracing. Our study had ethical approval from the Riverside research committee and informed consent

was obtained from the patients. Table 1 lists the demographic details of the patient and control groups.

Gene expression studies

RNA isolation and cDNA preparation

Plaques, aneurysm specimens, and control arteries were immediately snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted from approximately 30 mg of pulverised frozen tissue with the RNeasy mini or lipid tissue mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

For cDNA synthesis, total RNA (100 ng) was transcribed with a first strand cDNA synthesis kit for reverse transcription polymerase chain reaction (PCR) (Roche, Welwyn Garden City, Hertfordshire, UK), according to the supplier's instructions.

Real time quantitative reverse transcription PCR (Taqman®)

Primers and probes for Taqman® analysis of ABCA1 mRNA were designed to span two adjacent exons with PrimerExpress software (PE Applied Biosystems, Oxford, UK).

Tables 2 and 3 list the primers and probes for all genes investigated.

Single tube Taqman analysis was performed on an ABI Prism 7700 sequence detection system with 300nM of forward and reverse primers in the presence of a 200nM 5'FAM–3'TAMRA tagged probe for ABCA1, COX-2, MMP-9, and PPARγ and 900nM of forward and reverse primers in the presence of a 300nM 5'FAM–3'TAMRA tagged probe for LXRα. The internal standard was β actin mRNA, assayed with commercially supplied reagents (PE Applied Biosystems). Reactions were carried out in duplicate and contained 5 µl of undiluted cDNA in a total volume of 25 µl.

Quantitation

The amount of mRNA in cells was calculated according to the relative standard curve method described in the PE user bulletin number 2. Target quantity was calculated from the standard curve and normalised to β actin.

Statistical methods

The SAS 8.1 program package was used for statistical evaluations (SAS Version 8.e. SAS Institute Inc, Cary, USA. SAS 1999). For statistical analysis of mRNA expression, the Δ Ct (cycle threshold) values (Ct target gene − Ct β actin) were used. Samples were tested for normality by means of the UNIVARIATE procedure and the Shapiro-Wilk W test. The null hypothesis was rejected in none of the samples. We used analysis of variance to test differences among the control group, plaques, and arteries; pair wise significant differences were established using Bonferroni corrected t tests. Sex differences between the patient and control groups were assessed using the χ^2 test (FREQ procedure). We tested the significance of the Pearsons correlation coefficient to evaluate the relations between normalised ABCA1 and normalised MMP-9. A p value < 0.05 was considered significant.

Table 3 Primers for the genes investigated

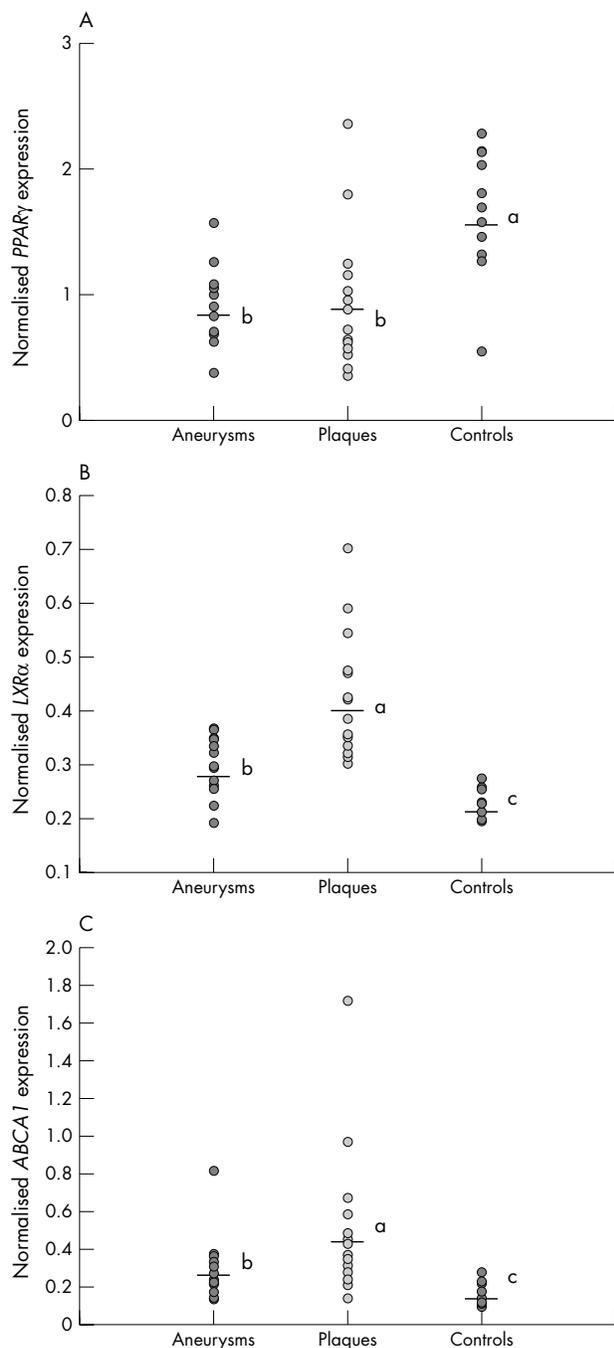| Genes	Forward primer (5'–3')	Reverse primer (5'–3')
ABCA1	GGGAGGCTCCCGGAGTT	GTATAAAAGAAGCCTCCGAGCATC
LXRα	CAAGTGTTTGCACTGCGTCT	CAGGAATGTTTGCCCTTCTC
PPARα	CCAGTGGTTGCAGATTACAAGTCTG	TTGTAGAGCTGAGTCTTCTCAGAATAATAAG
COX-2	GCCCTTCCTCCTGTGCC	AATCAGGAAGCTGCTTTTTACCTTT
MMP-9	GACGACCGGTTTGGCTTCT	AGGGTTTCCCATCAGCATTG

Figure 1 Expression of PPARγ, LXRα, and ABCA1 mRNA in aneurysms, plaques, and control arteries. Quantitative, real time polymerase chain reaction was performed on total RNA extracted from aneurysms (n = 16), plaques (n = 16), and control arteries (n = 12). Expression of PPARγ, LXRα, and ABCA1 mRNA was normalised to the β actin housekeeping gene. Bars indicate mean values of each group; a, b, c: means without a common letter are significantly different (p < 0.05). (A) PPARγ expression in aneurysms, plaques, and controls. (B) LXRα expression in aneurysms, plaques, and controls. (C) ABCA1 expression in aneurysms, plaques, and controls.

RESULTS

Expression of genes implicated in lipid efflux (ABCA1, PPARγ, LXRα)

Real time quantitative PCR was used to determine PPARγ mRNA levels in plaques and control arteries. PPARγ expression was significantly lower in occlusive and ectatic atherosclerotic tissues, compared with arterial control tissue (fig 1A;
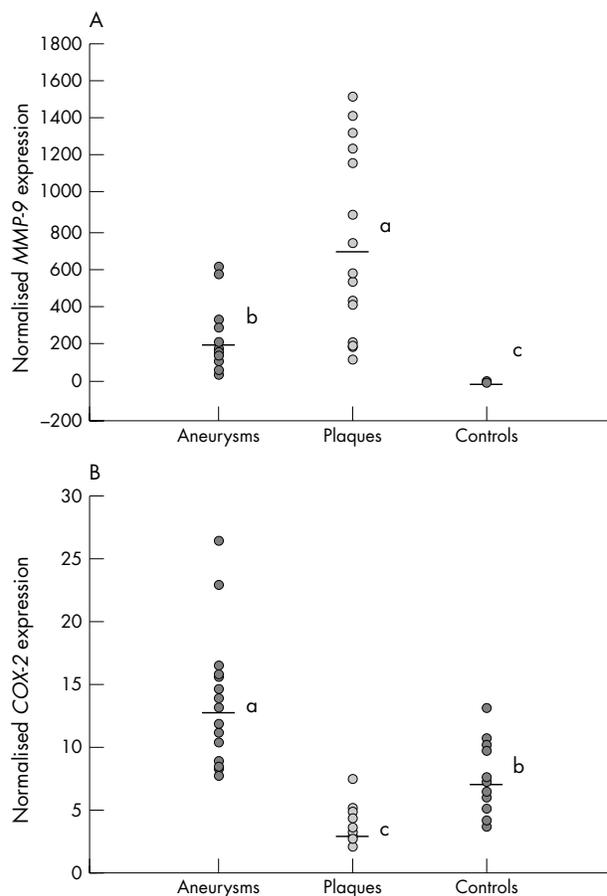

Figure 2 Expression of COX-2 and MMP-9 mRNA in aneurysms, plaques, and control arteries. Quantitative, real time polymerase chain reaction was performed on total RNA extracted from aneurysms (n = 16), plaques (n = 16), and control arteries (n = 12). Expression of COX-2 and MMP-9 mRNA was normalised to the β actin housekeeping gene. Bars indicate mean values of each group; a, b, c: means without a common letter are significantly different (p < 0.05). (A) MMP-9 expression in aneurysms, plaques, and controls. (B) COX-2 expression in aneurysms, plaques, and controls.

p < 0.001); LXRα and ABCA1 mRNA was significantly increased in both tissues (fig1 B,C; p < 0.01). LXRα and ABCA1 mRNA was markedly increased in occlusive compared with ectatic disease (p < 0.01 and p < 0.05, respectively).

Expression of genes implicated in inflammation and matrix degradation (MMP-9, COX-2)

MMP-9 expression was significantly higher in ectatic and stenotic arteries (fig 2A; p < 0.01). The highest expression was found in occlusive disease. COX-2 expression was significantly higher in aneurysm tissue (fig 2B; p < 0.01), whereas it was markedly reduced in atherosclerotic plaques compared with control tissue (p < 0.01).

Association between ABCA1 and MMP-9

ABCA1 and MMP-9 mRNA expression was associated in both occlusive and ectatic atherosclerotic tissues (fig 3A,B; r = 0.71 and 0.78 for plaques and aneurysms, respectively; p < 0.01).

DISCUSSION

There is much interest in PPARγ in terms of atherosclerosis because of its potential beneficial effects. It is expressed by all major cells of the vasculature, including endothelial cells,
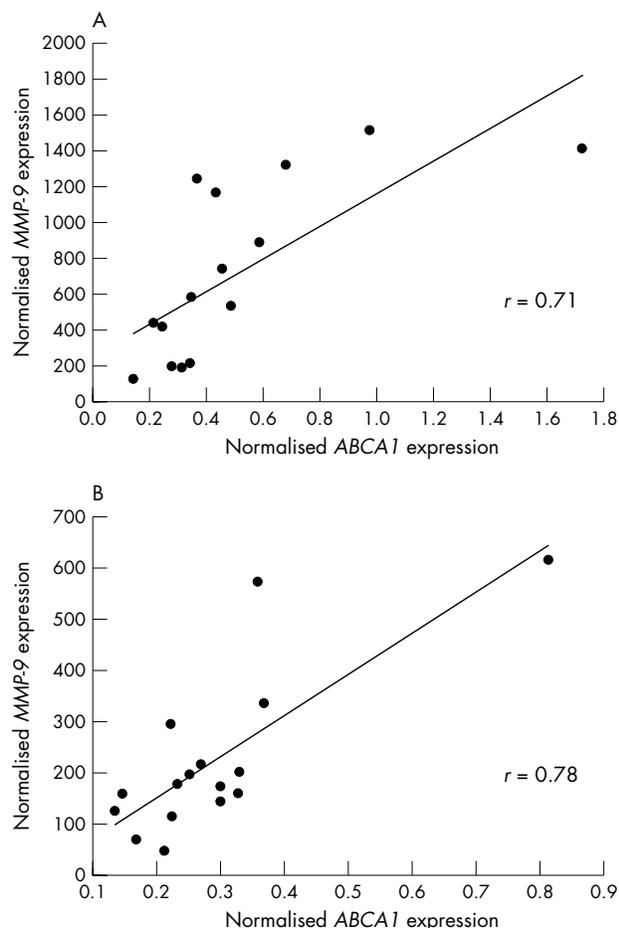
Figure 3 Association between *ABCA1* and *MMP-9* mRNA values in plaques and aneurysm tissue. *ABCA1* and *MMP-9* expression in (A) plaques and (B) aneurysm tissue was measured by real time polymerase chain reaction and normalised to β actin.

vascular smooth muscle cells, and monocytes/macrophages.[29] This receptor can be therapeutically targeted using the thiazolidinediones—a group of drugs such as pioglitazone, rosiglitazone, and troglitazone—agents that have recently been used in the treatment of type 2 diabetes and have proved to be very effective in reducing insulin resistance.[30–33]

It has been shown that PPARγ activation inhibits angiogenesis, which plays an important role in plaque progression and aneurysm formation,[34] and stimulates the release of nitric oxide from endothelial cells,[35] which is crucial for the maintenance of normal vascular physiology. Furthermore, drugs like thioglitazones have been found to inhibit intimal hyperplasia[36] and promote lipid efflux, in addition to reducing inflammation.[37] The clinical relevance of this has been demonstrated by the fact that less neointima formation was seen after coronary artery stent placement in patients with type 2 diabetes when they were treated with troglitazone.[38] In vivo studies using LDL receptor knockout mice have shown that PPARγ agonists reduced the development of atherosclerotic lesions.[15 39]

In our present study, we found reduced expression of *PPARγ* in human atherosclerotic tissues, both occlusive and ectatic, when compared with normal arterial controls, whereas *ABCA1* and *LXRα* expression was significantly upregulated in both types of disease. We have previously reported that ABCA1 protein was low in carotid atherosclerotic plaques, despite increased mRNA expression.[40] We hypothesised that reduced ABCA1 protein leads to an

oxysterol rich plaque microenvironment, which in turn stimulates LXRα, with consequent upregulation of the *ABCA1* gene.

This could explain the high expression of *LXRα* and *ABCA1* mRNA in these tissues. The variable degrees of *ABCA1* and *LXRα* upregulation in both occlusive and aneurismal disease could be attributed to the difference in the availability of ligands activating these genes. The reduced expression of *PPARγ* in these tissues could potentially result from the increased amount of cytokines in the plaque microenvironment.[41]

Expression of *MMP-9*, which is implicated in the degradation of the plaque fibrous cap, was found to be significantly raised in both types of diseased tissue, but more so in occlusive disease.[11 42] Although its role in aneurysm formation is not clear, MMP-9 has been extensively studied in the context of plaque pathophysiology.[42 43] Surprisingly *COX -2* expression was decreased in atheromatous plaques in our study, whereas expression was significantly higher in aneurysms. Oxidised LDL has been reported to inhibit COX-2 in human macrophages in in vitro studies,[44] suggesting that the impact of macrophage COX-2 may be attenuated in advanced atherosclerotic lesions.[45]

Moreover, oxidised LDL may reach higher concentrations in the plaque than in the aneurysm wall. This also seems to reflect the recent findings that therapeutic COX-2 inhibition may not be beneficial in stabilising the plaque, considering the fact that most of these plaques were symptomatic.[46 47]

"Our study underlined the potential link between genes involved in lipid efflux and matrix degradation"

We found that *ABCA1* mRNA expression correlated with *MMP-9* expression in both aneurysmal and occlusive specimens. Low concentrations of ABCA1 protein in atherosclerotic plaques[40] could account for the increased *ABCA1* mRNA values in these tissues.

Proteases such as calpain have been reported to be involved in ABCA1 protein degradation.[48] MMPs are known to degrade non-extracellular matrix proteins in addition to matrix proteins,[49] and were also found to correlate with calpain in in vitro studies.[50 51] This raises the possibility that common protein degradation pathways involving MMP-9 and calpain may be involved in ABCA1 protein degradation, thus promoting matrix degradation and at the same time reducing lipid efflux. In our study, a large proportion of patients in the carotid endarterectomy and aneurysm groups had comorbid conditions and were on aspirin and statins. Conditions such as diabetes mellitus can potentially decrease *ABCA1* expression[52 53]; however, the effect of hypertension on the expression of these genes has not been reported. Data on the influence of statins on *ABCA1* expression are conflicting,[54 55] although a positive effect on *PPARγ* expression and suppression of *MMP-9* and *COX-2* has been reported in various in vitro studies.[56 57]

PPARγ is known to suppress the synthesis of both COX-2 and MMP-9.[58–60]

Considering the low expression of *PPARγ* in these specimens, it is tempting to speculate that PPARγ upregulation through pharmacological means using thiazolidinediones or synthetic ligands could potentially be beneficial in increasing lipid efflux through LXRα and ABCA1 and reducing inflammation through the inhibition of COX-2 and MMP-9, thus stabilising the atherosclerotic plaque.

In conclusion, our observational study revealed low expression of *PPARγ* in ectatic and occlusive disease and underlined the potential link between genes involved in lipid efflux and matrix degradation. The interesting finding that reduced *PPARγ* expression is seen in atherosclerotic tissues

Take home messages

- We found that the expression of *PPARγ* (peroxisome proliferator activated receptor γ; a modulator of atherosclerosis with anti-inflammatory and anti-protease activity) was low in ecstatic and occlusive atherosclerotic disease

- These results suggest that the upregulation of PPARγ, by means of thiazolidinediones or synthetic ligands, could be beneficial in the context of treating atherosclerosis

- Upregulation of PPARγ may upregulate lipid efflux through ATP binding cassette transporter A1 and inhibit matrix degradation through inhibition of matrix metalloproteinase 9

raises the possibility that upregulation of this pathway may be beneficial in the context of treating atherosclerosis.

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