Reduction of monocyte chemoattractant protein 1 and macrophage migration inhibitory factor by a polyphenol-rich extract in subjects with clustered cardiometabolic risk factors

Lysette N. Broekhuizen[1], Diederik F. van Wijk[1], Hans Vink[2], A. Stalmach[3], A. Crozier[3], B. A. Hutten[1], John J. P. Kastelein[1], Paul G. Hugenholtz[4], Wolfgang Koenig[5] and Erik S. G. Stroes[1]*

[1]*Department of Vascular Medicine, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands*
[2]*Department of Physiology, University of Maastricht, Maastricht, The Netherlands*
[3]*Centre for Population and Health Sciences, University of Glasgow, Glasgow, UK*
[4]*Department of Cardiology, Erasmus University, Rotterdam, The Netherlands*
[5]*Department of Internal Medicine – Cardiology, University of Ulm Medical Center, Ulm, Germany*

Abstract

Inflammation is a hallmark of the metabolic syndrome, which also contributes to a pro-atherogenic state. NF-κB activation, a critical step in regulating inflammatory reactions, can be inhibited by polyphenol (PF) extracts, at least *in vitro*. In the present study, we set out to study whether a PF-rich extract could attenuate the chronic inflammatory state and/or an acute immune response *in vivo* in subjects with clustered metabolic risk factors. A commercially available, PF-rich extract (500 mg daily) or placebo was administered for 4 weeks to thirty-four subjects with two or more metabolic risk factors using a randomised, double-blind, cross-over design. During the final study visit, an acute inflammatory challenge (lipopolysaccharide (LPS) 1 ng/kg body weight) was administered to a random subgroup of subjects (PF-rich extract (*n* 12) and placebo (*n* 12)). The PF-rich extract modestly reduced the inflammatory chemokines monocyte chemoattractant protein 1 (MCP-1) and macrophage migration inhibitory factor (MIF) (MCP-1 -6.5% (PF, median 116 (interquartile range 97–136) pg/ml *v.* placebo, median 124 (interquartile range 105–153) pg/ml; $P<0.05$); MIF -10.8% (PF, median 2512 (interquartile range 1898–3972) pg/ml *v.* placebo, median 2814·5 (interquartile range 2296–3852) pg/ml; $P<0.05$); however, other measured markers of inflammation and cardiometabolic disease, such as C-reactive protein, IL-6, HDL-cholesterol, adiponectin and oxidised LDL, remained unaffected. Following the LPS challenge, we found a statistically significant 48% reduction of MCP-1 production in the PF-rich extract group (*n* 12) *v.* placebo (*n* 12) over 6 h (PF 766 (sd 155) *v.* placebo 1466 (sd 989) ng/ml; $P<0.05$, area under the curve). In conclusion, short-term oral administration of the PF-rich extract caused a modest anti-inflammatory effect in subjects with clustered metabolic risk factors. Further dose-ranging studies are needed to evaluate whether and to what extent PF-rich extracts can be used to reduce the pro-inflammatory state in subjects with metabolic diseases at increased cardiovascular risk.

Key words: Flow-mediated dilation: Polyphenols: Inflammation: Cardiometabolic risk factors: Monocyte chemoattractant protein 1

Inflammation plays a critical role during all stages of the atherogenic process, ranging from the initiation of endothelial dysfunction to the onset of a cardiovascular event[1]. In agreement with this, elevated levels of pro-inflammatory chemokines and cytokines have been associated with increased cardiovascular risk[2–5]. Novel interventions that attenuate inflammation are being evaluated as an add-on strategy in patients at increased cardiovascular risk[6]. The metabolic syndrome, a major risk factor for CVD, is characterised by a chronic low-grade inflammatory state[7–9]. The latter has been attributed to inappropriate adipocyte enlargement accompanied by increased stress in the endoplasmatic reticulum of the adipocytes, leading to activation of NF-κB and subsequent inflammatory activation as attested by an increased production of IL-6, TNF-α, macrophage migration inhibitory factor (MIF) and monocyte chemoattractant protein 1 (MCP-1)[10,11]. These pro-inflammatory cytokines modulate the paracrine function of the adipocytes, further contributing to systemic inflammation and insulin resistance in patients with obesity[10,12].

Polyphenols (PF) are chemical substances found in plants containing two or more phenol groups. *In vitro*, PF have

been shown to inhibit Toll-like receptor (TLR)-mediated inflammatory responses via the MyD88-independent TLR3- and TLR4-signalling pathways[13], diminish NF-κB activation and reduce the production of downstream chemokines and cytokines[13,14]. Antioxidative and anti-thrombotic effects have also been reported. Consistent with the anti-atherogenic effects *in vitro*, PF administration has been shown to attenuate atherosclerotic lesion formation in a variety of animal models[15–18]. In human subjects, however, the results of PF administration are heterogeneous[18–22]. Whereas a single study did report a beneficial effect of PF on the carotid intima-media thickness in patients at increased cardiovascular risk[23], the true impact of PF ingestion for CVD needs confirmation in larger clinical trials. To date, the bulk of studies observing an effect of PF ingestion is based on epidemiological studies with very few randomised, placebo-controlled trials[24–26].

In the present randomised, placebo-controlled, double-blind study, we evaluated the impact of daily oral intake of 500 mg of PF-rich extract for 4 weeks on inflammatory markers in thirty-four subjects with a clustering of cardiometabolic risk factors. In addition, we challenged a random subgroup (*n* 24) with low-dose endotoxin to also evaluate the effect of the PF-rich extract on the acute inflammatory response.

Methods

Participants

In the present study, thirty-four participants were recruited from the outpatient clinic of the Academic Medical Center (Amsterdam, The Netherlands) and through poster advertisement. The subgroup for the endotoxin challenge consisted of twenty-four participants who were randomly selected and asked for consent at the first study visit. Subjects were eligible to participate if two or more of the following criteria were present: a waist circumference of ≥102 cm for men or ≥88 cm for women, TAG levels ≥1·69 mmol/l, HDL-cholesterol ≤1·03 mmol/l for men or ≤1·29 mmol/l for women, a systolic blood pressure ≥130 mmHg, a diastolic blood pressure ≥85 mmHg and a glucose level ≥6·1 mmol/l, according to the Adult Treatment Panel III criteria (attributed to the National Cholesterol Education Program/National Heart, Lung, and Blood Institute)[27]. During the study period, participants were instructed to refrain from wine and grape-containing beverages, large amounts of tea, fruit juice and dark chocolate. The use of lipid-lowering medication or antioxidants was not allowed throughout the study. Subjects were excluded if they reported CHD, stroke, malignancies or chronic inflammatory diseases in their medical history. Subjects who were current smokers or known with alcohol abuse were excluded from the study.

Design

The study was designed as a double-blind, placebo-controlled, randomised, cross-over trial. During the screening visit,

2 weeks before the first study visit, blood was withdrawn for measurement of baseline parameters. Participants were randomised to daily 500 mg PF-rich extract obtained from grapes and apples for 4 weeks or placebo. The PF-rich extract contains a total of thirty-six (poly) phenolic compounds, including flavan-3-ols (monomers and oligomers up to a degree of polymerisation of 10), flavonols (mainly quercetin derivatives and myricetin), chlorogenic acids (5-caffeoylquinic and 4-*p*-coumaroylquinic acids), stilbenes (*trans*-resveratrol), dihydrochalcones (phloretin and derivatives) and anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin derivatives). The total amount of (poly) phenolic compounds in the extract is 507 (SD 4) mg/g of power. A detailed analysis section of the PF extract is presented in the supplementary material (available online at http://www.journals.cambridge.org/bjn).

Following a 4-week washout period, participants were switched to placebo or PF-rich extract, respectively. The daily amount of PF-rich extract and placebo was dosed in capsules of 250 mg. Participants were asked to take two capsules a day in the morning. At the end of each treatment period, blood was drawn for laboratory testing, including total cholesterol, LDL-cholesterol, HDL-cholesterol, glucose, glycated Hb, C-reactive protein, Hb and creatinine. Blood pressure was measured and endothelial function (flow-mediated dilatation (FMD) by ultrasound) was assessed (see below). All measurements were performed after an overnight fast. At the end of the second treatment period, a subgroup of twenty-four participants (fifteen men and nine women), who consented to additional investigation, was challenged with a low dose of endotoxin derived from *Escherichia coli* at a dose of 1 ng/kg of body weight of endotoxin (*E. coli* lipopolysaccharide (LPS) (O113;H10), 10·00 EU/vial, pharmaceutical development section (PDS) no. 67801, lot no. 67466 (production date 4 January 1997); Cape Cod, Inc./National Institutes of Health, Bethesda, MD, USA)[28–30]. Subsequently, blood was withdrawn from the contralateral antecubital vein at 1, 3, 4, 6 and 8 h. Vital signs were measured at regular intervals, every 15 min in the first 3 h. Incidence, time and severity of symptoms were recorded by the study physician. FMD measurements were performed at baseline and 4 h after the LPS challenge. Approval for the present study was obtained from the internal review board of the Academic Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki. All participants gave written informed consent.

Laboratory measurements

Baseline serum concentrations of total cholesterol, HDL-cholesterol and TAG were measured in fresh serum samples by standard enzymatic methods (Roche Diagnostics, Basel, Switzerland). LDL-cholesterol concentrations were calculated using the Friedewald formula. Glucose was assessed using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi, Mannheim, Germany). Glycated Hb was measured by HPLC (Reagens; Bio-Rad Laboratories, Veenendaal, The Netherlands) on a Variant II (Bio-Rad Laboratories). Plasma aliquots

were snap-frozen and stored at −80°C for shipment to the Biomarker Laboratory at the University of Ulm, Germany. Plasma C-reactive protein levels were measured with a high sensitivity latex-enhanced nephelometric assay on a BN II analyser (Dade Behring, Marburg, Germany). TNF-α, IL-6, adiponectin, oxidised LDL, MCP-1 and MIF levels were measured using commercially available ELISA kits (R&D Systems, Abingdon, Oxford, UK). The intra- and inter-assay CV of quality-control test sera were <10 and <20%, respectively.

Flow-mediated dilatation measurements

Each patient underwent measurement of FMD of the left brachial artery by B-mode ultrasound imaging using an Acuson Aspen (Siemens, Mountain View, CA, USA) ultrasound system with an L7, 5–10 MHz linear array broadband transducer. The FMD of all thirty-four participants was measured after each treatment period. The FMD of the twenty-four participants receiving an additional LPS challenge was also measured 4 h after the LPS challenge. Patients were instructed to refrain from food, alcohol and any other drink except water before the measurements. A blood pressure cuff was placed on the left forearm from the medial epicondyle downwards. The scan of the left brachial artery started with 1 min of continuous baseline recording, followed by 5 min of forearm ischaemia, induced by inflating a vascular pressure cuff to 250 mmHg. Hyperaemic blood flow was induced by deflating the cuff, followed by 3 min of continuous ultrasound recording. A semi-automated image analysis of scans was performed off-line by an experienced image analyst using dedicated software (Brachial Analyzer; MIA Vascular Tools, Coralville, IA, USA). Images were blinded for treatment and visit. The average baseline diameter and the maximum post-cuff deflation diameter were used to calculate the percentage of flow-mediated vasodilation (%FMD), which was defined as follows:

$$\%FMD = 100 \times (\text{maximum post-cuff deflation diameter}$$
$$- \text{baseline diameter})/\text{baseline diameter}.$$

Statistical analysis

Data are presented as means and standard deviations when normally distributed and as medians with corresponding interquartile ranges for values with a skewed distribution. For the cross-over study, we used a repeated-measures mixed model, with time and treatment as fixed effects. An interaction term of time and treatment was used to test for possible carry-over effects. For the LPS intervention, we calculated the production of circulating mediators after the LPS challenge by the area under the curve from the time of the infusion ($t = 0$) until 6 h after the start of the challenge ($t = 6$). A paired-samples t test was used for normally distributed values, whereas a Wilcoxon rank test for paired measurements was employed to test for skewed variables. Analyses were performed with

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). A P value <0·05 was defined as statistically significant.

Results

### Baseline characteristics of the participants

Baseline characteristics of the study participants are listed in Table 1. Of the thirty-four participants, 44% had two Adult Treatment Panel III factors, whereas 56% had three Adult Treatment Panel III factors (attributed to the National Cholesterol Education Program/National Heart, Lung, and Blood Institute)[27] of the metabolic syndrome. The prevalence of risk factors was distributed as follows: increased waist circumference 88%, hypertriacylglycerolaemia 50%, low-HDL-cholesterol levels 27%, elevated blood pressure 88% and elevated plasma glucose levels 29%. The average age was 58·3 (SD 7·9) years. Baseline characteristics of the participants were as follows: BMI 31·9 (SD 4·8) kg/m², systolic blood pressure 147 (SD 18·3) mmHg, diastolic blood pressure 89 (SD 9·3) mmHg, total cholesterol 5·6 (SD 1·1) mmol/l, LDL-cholesterol 3·3 (SD 0·9) mmol/l, HDL-cholesterol 1·3 (SD 0·4) mmol/l, TAG 1·76 (1·38–3·23) mmol/l, glucose 5·6

Table 1. Markers for cardiovascular risk and inflammation according to the treatment period of all thirty-four participants

(Mean values, standard deviations, medians and interquartile ranges)

	Placebo		Polyphenols		
	Mean	SD	Mean	SD	P
Age (years)	58·3	7·9	58·3	7·9	NS
BMI (kg/m²)	31·9	4·9	31·6	5·2	NS
SBP (mmHg)	142·5	16·8	143·0	14·7	NS
DBP (mmHg)	86·1	10·3	87·0	8·9	NS
Total cholesterol (mmol/l)	5·57	1·29	5·71	1·19	NS
LDL-cholesterol (mmol/l)	3·39	1·16	3·56	1·11	<0·05
HDL-cholesterol (mmol/l)	1·33	0·31	1·32	0·33	NS
TAG (mmol/l)					NS
Median	1·76		1·88		
Range	1·33–2·39		1·21–2·41		
Glucose (mmol/l)					NS
Median	6·0		6·1		
Range	5·6–6·8		5·7–6·9		
HbA1C (%)	5·8	0·4	5·8	0·4	NS
CRP (mg/l)					NS
Median	2·37		2·43		
Range	1·36–3·94		1·19–5·03		
Oxidised LDL (nmol/ml)					NS
Median	91·0		91·5		
Range	77·1–118		79–116		
IL-6 (pg/ml)					NS
Median	1·55		1·38		
Range	1·21–1·79		1·1–1·83		
MCP-1 (pg/ml)					<0·05
Median	124		116		
Range	105–153		97–135·8		
MIF (pg/ml)					<0·05
Median	2814·5		2511·5		
Range	2296–3852·3		1898–3972		
Adiponectin (μg/ml)					NS
Median	8·0		8·5		
Range	6·3–10·8		5·3–11·8		

SBP, systolic blood pressure; DBP, diastolic blood pressure; HbA1C, glycated Hb; CRP, C-reactive protein; MCP-1, monocyte chemoattractant protein 1; MIF, macrophage migration inhibitory factor.
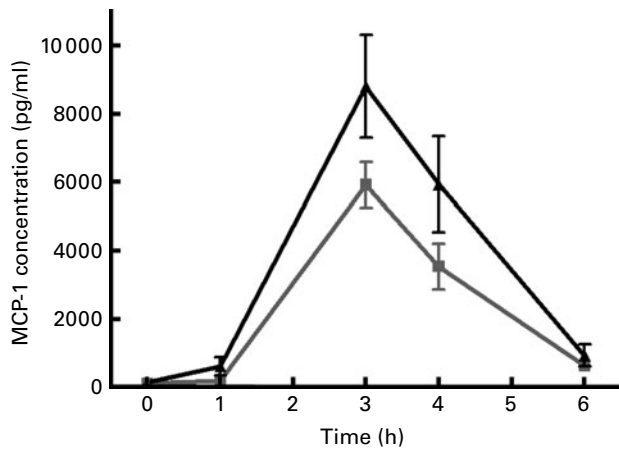
Fig. 1. Area under the curve (over 6 h of measurement) of monocyte chemoattractant protein 1 (MCP-1). Production of MCP-1 after treatment with polyphenol-rich extract (■) is significantly lower compared with treatment with placebo (▲); 766·1 v. 1465·5 ng/ml (P=0·04) (polyphenols (n 12) v. placebo (n 12)). Values are means, with their standard deviations represented by vertical bars.

(5·1–6·1) mmol/l, glycated Hb 5·8 (SD 0·5) % and C-reactive protein 2·1 (1·1–2·8) mg/l.

Effect of polyphenol-rich extract on inflammatory parameters

After 4 weeks, BMI and blood pressure were unaffected in both PF-rich extract and placebo groups (Table 1). No significant interaction term was found, indicating that no carry-over effects were present in the present study. We found a statistically significant reduction of 6·5 and 10·8 % for the MCP-1 and MIF levels, respectively, in the PF-rich extract group v. placebo. No changes were observed for other markers measured (Table 1). FMD was comparable between the groups (Figs. 1 and 2).

Effects of polyphenol-rich extract on lipopolysaccharide response

During the final study visit, twenty-four participants received an inflammatory challenge of LPS (1 ng/kg body weight). All participants showed a modest rise in temperature with concomitant side effects including chills, accelerated heart rate and headache. These symptoms did not differ between the PF-rich extract and placebo groups. Total MCP-1 production, expressed as area under the curve up to 6 h, was reduced in the PF-rich extract group compared with placebo. The ensuing peak increases in other measured inflammatory parameters were not significantly different (data not shown). FMD was significantly reduced 4 h after the LPS challenge (Figs. 1 and 2) in both groups. This attenuation was comparable in both PF-rich extract and placebo groups.

Discussion

In the present study, we show that the 4-week administration of 500 mg PF-rich extract was associated with a significant,

albeit modest, reduction in MCP-1 and MIF levels in subjects with clustered metabolic risk factors. MCP-1 production (area under the curve over 6 h) following an LPS challenge was significantly reduced in the PF group compared with placebo, whereas no differences were observed for the other measured inflammatory cytokines. The present findings confirm a modest anti-inflammatory effect of PF-rich extracts in subjects with clustered metabolic risk factors in vivo. Yet, in view of the modest effect on isolated inflammatory parameters, larger studies are needed with prolonged follow-up in order to establish potential clinical relevance of this observation.

Polyphenol and inflammatory markers

PF-rich extracts from various food sources, including alcoholic beverages made from grapes, have been shown to exert a variety of beneficial effects, including antioxidative, antithrombotic and vasodilatory effects[10,19,31–38]. In addition, PF-rich extracts have been reported to reduce the inflammatory response, particularly for TLR3- and TLR4-specific ligands. Since the metabolic syndrome has been associated with a low-grade inflammatory state in which a diet-related, postprandial increase of endotoxins may play a role[39], we focused on patients with clustered metabolic risk factors to assess a potential anti-inflammatory effect of PF in vivo. Following 4 weeks of PF-rich extract administration, a significant, albeit modest, reduction in circulating levels of MCP-1 and MIF was observed. This finding is in correspondence with previous reports corroborating the anti-inflammatory
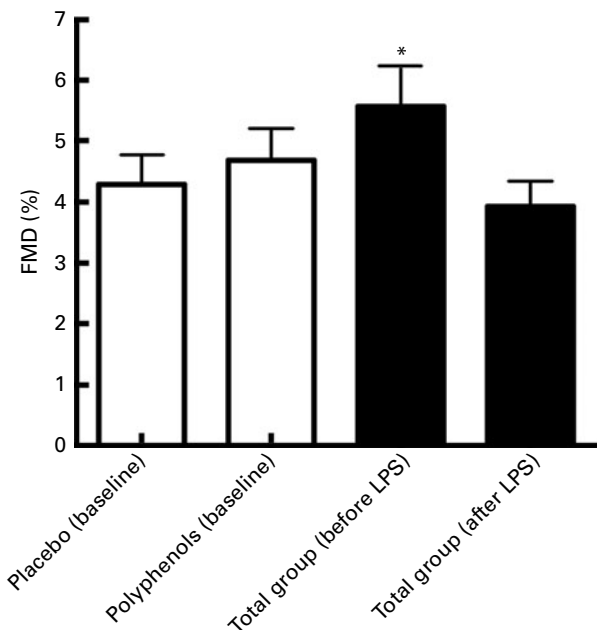
Fig. 2. Flow-mediated dilatation (FMD) of the left brachial artery was not significantly different between the placebo and polyphenol-rich extract groups; 4·28 (SD 0·49) and 4·69 (SD 0·52) (P=NS), respectively (n 24). FMD did show a significant difference before and 4 h after the lipopolysaccharide (LPS) infusion in the subgroup; 5·56 (SD 0·7) and 3·84 (SD 0·43) (*P<0·05, n 14. Values are means, with their standard deviations represented by vertical bars.

capacity of PF in animal models[40,41]. Similarly, the phenolic compound resveratrol has been shown to inhibit NF-κB transcription in vitro[13,42]. In healthy volunteers, consumption of the PF extract in alcoholic beverages has also been reported to reduce NF-κB activation as well MCP-1 plasma levels during a high-fat diet[43]. Both MCP-1 and MIF are considered to be important pro-inflammatory cytokines associated with increased cardiovascular risk. MCP-1 is an important chemokine, attracting monocytes to the sites of endothelial injury, and both MCP-1 serum levels and MCP-1 genotype have been associated with CVD[44]. Mice receiving pharmacological blockade of MCP-1 and mice deficient in MCP-1 develop less atherosclerosis compared with placebo-treated or wild-type mice[45,46]. Similarly, MIF has been associated with atherogenesis as well as risk factors associated with the development of metabolic disorders such as obesity and insulin resistance[47]. MIF is a pleiotropic cytokine that acts, among others, as a pro-inflammatory cytokine[48]. Both MIF and MCP-1 play a crucial role in the chemotaxis of monocytes into the adipose tissue, which is a key step in the progression towards 'dysfunctional' adipose tissue[47,49,50], characterised by a dysregulation of adipocyte paracrine function. We did not observe a difference in other markers associated with a pro-atherogenic state between the PF-rich extract and placebo groups. Based on the present findings, one might speculate whether PF-rich extracts can have a positive effect on 'dysfunctional' fat tissue in patients with the metabolic syndrome after long-term exposure[10].

Polyphenol and the inflammatory reaction following lipopolysaccharide challenge

Consistent with the effect of the PF-rich extract on the chronic inflammatory state, a challenge with LPS resulted in a reduced MCP-1 area under the curve over 6 h in the PF-rich extract group compared with placebo. The other measured inflammatory markers did not differ. Monagas et al.[51] recently described that pre-treatment of peripheral blood mononuclear cells with certain phenolic metabolites induced a reduction of TNF-α, IL-6 and IL-1β production after LPS stimulation by more than 80%. Another in vitro study has observed differential effects between the various phenolic compounds that were used. For instance, a reduction of IL-1β could be detected for addition of oleuropein glycoside, present in olive oil, but not for other PF used. No effect was observed on TNF-α or IL-6[52], the latter finding being more similar to the results of the present study. It has been suggested that not all PF have comparable anti-inflammatory characteristics and that different PF have specific anti-inflammatory properties, which could in part explain the heterogeneous results between previously published studies[53].

Limitations

The results of the present study warrant several comments. First, a treatment period of 4 weeks is a relatively short period and the dose of 500 mg/d may be suboptimal as bioavailability of many polyphenolic substances has been reported to be poor[54]. Thus, large amounts of PF may have

to be ingested in order to obtain beneficial effects. It has to be taken in account that this characteristic limits the clinical application of these compounds. Second, the PF-rich extract used is a mixture of compounds, which includes various substances and may even enclose potential non-polyphenolic compounds. This is a first exploratory study to investigate the effect of a PF-rich extract on both acute and chronic inflammatory reactions in obese participants with multiple cardiometabolic risk factors. Markedly, despite the short duration of PF administration as well as the limited number of participants studied, we were able to detect a significant effect on both basal MCP-1 and MIF plasma levels, as well as the acute MCP-1 response following an endotoxin challenge.

Conclusions

The present study shows that oral ingestion of a PF-rich mixture extracted from apples and grapes decreases circulating pro-inflammatory cytokines, involved in both the pathogenesis of adipocyte dysfunction and atherogenesis. Considering the important role for the infiltration of monocytes into fat tissue and the subsequent inflammatory response, PF may have a potentially beneficial effect on the development and/or progression of CVD in patients with clustered metabolic risk factors. However, this needs further confirmation in larger trials investigating the optimal dose and the long-term results of PF administration on the development and progression of CVD.

Acknowledgements

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