TABLE 1. Effect of non-surgical periodontal treatment on glycemic control in people with type 2 diabetes: meta-analyses published as of July 19, 2014.

Meta-analysis	# studies	# RCTs	Pooled # subjects	HbA_{1c} change	95% CI	*p*-value
Janket et al (2005)[2]	5	1	268	−0.66%[a]	−2.2; 0.9	ns
Darre et al (2008)[3]	9	9	485	−0.46%[c]	−0.82; −0.11	0.01
Teeuw et al (2010)[4]	5	3[b]	180	−0.40%[c]	−0.77; −0.04	0.03
Simpson et al (2010)[5] Cochrane Review	3	3	244	−0.40%	−0.78; −0.01	0.04
Sgolastra et al (2013)[6]	5	5	315	−0.65%	−0.88; −0.43	<0.05
Engebretson and Kocher (2013)[7]	9	9	775	−0.36%	−0.54; −0.19	<0.0001
Liew et al (2013)[8]	6	6	422	−0.41%	−0.73; −0.09	0.013

CI: confidence interval; HbA_{1c}: glycated hemoglobin; ns: non-significant; RCT: randomized controlled trial.
[a]Weighted.
[b]Remaining two non-RCT studies are clinical controlled trials.
[c]Standardized mean difference.

periodontitis. These findings do not support the use of nonsurgical periodontal treatment in patients with diabetes for the purpose of lowering levels of HbA_{1c}."

COMMENTARY AND ANALYSIS

When developing clinical recommendations and guidelines, it is important to consider the highest levels of evidence, which are typically derived from high-quality systematic reviews of high-quality RCTs that are sufficiently powered and well conducted to provide definitive evidence. However, individual large multi-center trials are often perceived by the busy reader to independently deliver definitive answers to the research question posed. Hence, the potential impact of the RCT discussed in this review[1] is significant because it will be considered to provide a higher level of evidence than previous systematic reviews of prior smaller studies. The majority of available studies have reported improvements in glycemic control (measured as HbA_{1c}) in people with type 2 diabetes after non-surgical periodontal therapy. HbA_{1c} measures long-term blood sugar levels over the lifespan of the red blood cell, weighted to the last 2 to 3 months. All seven systematic reviews and meta-analyses published conclude that such therapy does lead to improvements in glycemic control. They calculated similar magnitudes of HbA_{1c} improvement, ranging from 0.36 to 0.65 percentage points (Table 1). This reported impact is similar to that expected from adding a second oral antidiabetes medication to metformin and is therefore of clinical significance in the management of diabetes.

The US multi-center RCT under review[1,9] was anticipated to be a "definitive" study, unlike its smaller predecessors. Given the increasing global epidemic of type 2 diabetes and the need for novel approaches to manage and/or prevent diabetes and its complications, this multi-center study conducted in partnership with the funding agency, the US National Institute of Dental and Craniofacial Research (NIDCR), is very important because its findings are likely to influence the current scientific knowledge base, as well as evidence-based policy-making and clinical practice in many countries.

The basic goal underlying these intervention studies in persons with type 2 diabetes is to reduce the local microbial burden to a level sufficient to lead to clinically meaningful improvements in periodontal health. If successful, the systemic exposure and subsequent inflammatory burden would be reduced, which would in turn decrease long-term blood glucose levels, measured as HbA_{1c}. However, clinically meaningful improvement in periodontal health that is consistent with the standard of care reported in the world literature is an essential pre-requisite for a valid outcome. Otherwise, no effect on HbA_{1c} could reasonably be expected.

Concern 1: No Significant Effect of Periodontal Treatment Would Be Expected Because Baseline HbA_{1c} Levels Were Already Close to the Goal for Good Glycemic Control

Hyperglycemia defines diabetes, and its control is fundamental to diabetes care.[10] The goal for type 2 diabetes management is to attain and maintain an HbA_{1c} level of less than 7.0%, but lower (less than 6.5%) or higher (less than 8%) levels are acceptable for specific patient groups.[10] The HbA_{1c} value of 7.0% was selected as the lower limit for enrollment in the study, corresponding to average plasma glucose levels of 154 mg/dL (8.6 mmol/L). The upper limit was set at less than 9.0% (~212 mg/dL or 11.8 mmol/L). Nevertheless, 3.5% (9) of the test participants had HbA_{1c} levels of 9% or greater and 4.7% (12 subjects) had HbA_{1c} less than 7%, both in violation of the protocol's eligibility criteria. The baseline mean HbA_{1c} level was 7.84% in the treatment group (calculated

TABLE 2. Improvement in periodontal measures following non-surgical periodontal treatment: the reviewed RCT (n = 240; eTable 2[a])[1] *versus* literature-based expectations.

			Reported decrease	Expected decrease (non-diabetes)	
Periodontal parameter	**Baseline[1]**	**After treatment[1]**	**Engebretson (2013)[1]**	**Cobb (1996)[12] & (2002)[13]**	**Van der Weijden and Timmerman (2002)[14]**
Periodontal probing depth (PPD) [mean (mm)]	3.3 mm	2.9 mm	0.4 mm	• 1.29 mm (PPD 4–6 mm)[12] • 1.50 mm (PPD = 6 mm)[13] • 2.16 mm (PPD ≥ 7 mm)[12]	• 1.18 mm • 1.23 mm (mild periodontitis) • 2.26 mm (severe periodontitis)
Bleeding on probing (BOP) [proportion (%)]	60.6%	41.6%	19.0%	~45% (PPD 4.0–6.5 mm)[13]	n/a
Gingival index (GI)	1.4	1.0	0.4	1.0[13]	n/a

n/a: not available.
[a]Baseline figures differ slightly from Table 1 in the body of the report, which uses n = 257.[1]

from eTable 1) and 7.77% among controls (eTable 1). At baseline, 60.3% of the test group and 63.8% of the control group already had HbA_{1c} levels below 8.0%, leaving less than 40% with HbA_{1c} levels at 8.0% or greater. The potential for any intervention to improve glycemic control depends upon the baseline HbA_{1c} level: the higher the level, the greater the potential for improvement, and the lower the HbA_{1c} level, the more unlikely a further decrease becomes. Therefore the HbA_{1c} interval selected for inclusion in this study renders additional benefit from any adjunctive therapy less likely. With a mean baseline value of 7.8% and an upper limit for eligibility of less than 9%, the study subjects were already close to their target for glycemic control at enrollment.

Concern 2: No Conclusion Can Be Drawn Regarding Any Effect on Glycemic Control Because Periodontal Treatment Failed to Reach the Accepted Standard of Care

The reviewed study suffers from a second significant deficiency, namely the poor outcomes reported for the administered periodontal therapy. The clinical improvements in periodontal health are far below the expected standard of care and effectively negate the appropriateness of any conclusions based on this intervention. Fundamental to the appropriate interpretation of results from any periodontal intervention study is that the reductions in PPD, percent of sites with BOP (%BOP), plaque scores, and gains in clinical attachment are consistent with the world literature. When outcomes are below the expected standard of care, then the likelihood of incorrect conclusions being drawn is high.[11]

It is widely recognized that quoting statistically significant improvements in clinical outcomes that are based on the means or medians of hundreds of measures per patient is inappropriate unless the changes reported are of clinical significance and, most importantly, consistent with the literature. In Table 2, we have summarized the outcomes attained after non-surgical periodontal treatment in this study and compared them to the expected results in subjects without diabetes from systematic reviews. Table 3 displays the periodontal health status at baseline and at the end of the study. The periodontal treatment in this RCT resulted in poor levels of clinical improvement and left considerable inflammation (BOP) and very high dental plaque (infection) levels, which are highly likely to have precluded any reduction of HbA_{1c} in the test group and most likely explain the reported (but not statistically significant) increase in % HbA_{1c}.

It is unfortunate that these essential results are displayed exclusively in the online supplementary overview (eTable 2) and that the authors did not benchmark their results against the accepted literature.[12–14] A key question that should have been discussed is, "Why did the periodontal status of the individuals in the treatment arm not improve sufficiently and in a manner consistent with the periodontal outcomes in prior studies?" Because of the poor clinical improvement in periodontal conditions, the biological question of whether reducing periodontal infection/inflammation in a clinically signi-

TABLE 3. Periodontal health status in the treatment group ($n = 240$) at the beginning and at the end of the study (eTable 2[a]).[1]

Periodontal parameter	Baseline	End of study
Periodontal probing depth (PPD)		
PPD ≥4 mm [mean # sites/person]	50.9	30.6
PPD ≥5 mm [mean # sites/person]	28.8	15.7
PPD ≥4 mm [proportion sites (%)]	33.7%	20.1%
PPD ≥5 mm [proportion sites (%)]	19.0%	10.2%
Clinical attachment loss (CAL)		
CAL ≥4 mm [mean # sites/person]	59.7	44.0
CAL ≥5 mm [mean # sites/person]	35.7	23.6
CAL ≥4 mm [proportion sites (%)]	40.1%	29.8%
CAL ≥5 mm [proportion sites (%)]	6.6%	4.3%
Bleeding on probing (BOP) [proportion sites (%)]	60.6%	41.6%
Plaque score [% sites/person]	86.7%	72.1%

Study eligibility criterion: Moderate to advanced chronic periodontitis, defined as clinical attachment loss and probing depth of at least 5 mm in 2 or more quadrants.
[a]Baseline figures differ slightly from Table 1 in the body of the report[1] which uses n = 257.

ficant manner results in improved glycemic control cannot be answered by the results of this study.

The manner in which the authors portray the effect of the periodontal treatment implies that the treatment was successful, when in fact, it was not. The authors claimed: "*Using linear regression models, all periodontal clinical parameters improved significantly at 3 months and were sustained at 6 months in the treatment group but not in the control group.*" This statement directly leads the reader to believe that the periodontal treatment was successful. However, statistically significant improvements in periodontal outcome parameters are meaningless unless they have clinical relevance and are consistent with the standard of care and attainable results reported in the literature. These results are neither. At the end of the study, each person still had on average 30.6 sites (20.1%) with PPD 4 mm or greater, and half of those (15.7 sites or 10.2%) were 5 mm or deeper; 41.6% of all sites bled on probing; and 72.1% had plaque (Table 3).

Based on their periodontal status, it seems that a considerable proportion of the participants still had a level of periodontitis at the completion of the study that would render them eligible for enrollment into the study for periodontal therapy, based on their current level of periodontitis and the study's own inclusion criteria. The authors noted: "*improvements in plaque and bleeding scores were only modest and indicate that changing oral hygiene habits remains a challenge.*" However, previous intervention studies did manage to overcome this challenge and greatly improve periodontal health. Why could this large multi-center study not achieve sufficient plaque control consistent with the literature? The notion suggested by the authors that "*it is possible that periodontal inflammation and infection do not influence glycemic control. Indeed, the results of this trial indicate that glycemic control worsened, although not significantly, 6 months after study therapy*" assumes that periodontal treatment has been clinically effective. Because it was not, this statement is not supported by the study results.

Concern 3: Pronounced Obesity Would Mask Any Decrease in Inflammatory Response Caused by Successful Periodontal Treatment

A third significant problem is that the chronic, low-grade inflammatory state elicited by the prominent obesity in the treatment group (mean BMI 34.7(±7.5) kg/m^2) would have masked any anti-inflammatory effect of successful periodontal treatment. It is the decrease in inflammation due to periodontal infection that leads to the decrease in blood glucose levels, and thus we would not expect to be able to measure any significant decrease in glycated hemoglobin levels, even after successful periodontal treatment in such obese subjects. The Hiroshima Study demonstrated that HbA_{1c} levels improve by resolution of the periodontal infection-related systemic inflammation, but only in subjects with initially elevated levels of the acute-phase inflammatory marker C-reactive protein, measured with high sensitivity (hsCRP).[15] In fact, the initial hsCRP level is a significantly important independent variable influencing HbA_{1c} reduction rates, and the greatest reduction in HbA_{1c} level is experienced by the group with the highest hsCRP reduction following periodontal treatment.[15] Importantly, the subjects in the Hiroshima study were non-obese but had type 2 diabetes. An earlier US study called Atherosclerosis Risk in Communities (ARIC) already reported that, when the BMI of the subjects was in the 20s range, there was a predicted 2-fold difference in hsCRP between severe and no/mild periodontitis groups, but the difference decreased with increasing BMI and became negligible when BMIs reached 35 kg/m^2.[16] Furthermore, the Periodontitis and Vascular Events (PAVE) multi-centered trial demonstrated that systemic inflammation persisted among obese individuals following scaling and root planing.[17] In the current study, although the effect of periodontal therapy on the reduction in the systemic inflammatory burden was not reported, it is possible that most of the subjects were resistant to the

elimination of periodontal disease-related systemic inflammation due to the overwhelming influence of their obesity-related systemic inflammatory load.

Conclusion

Overall, this study actually raises more questions than it answers, which is an important outcome. Regrettably, it is not the definitive study for determining the effects of successful periodontal therapy on glycemic control in people with diabetes that it was anticipated to be.

There is no logical basis for expecting periodontal treatment that is not successful in controlling the periodontal infection and reducing inflammation to clinically acceptable levels to have any positive impact on glycated hemoglobin levels, in particular where obesity levels are high and glycemic control is close to target at enrollment. Consequently, the results of this large RCT are inconclusive and the results of this investigation do not permit meaningful statements to be made regarding whether or not *successful* non-surgical periodontal treatment contributes to glycemic control by decreasing HbA_{1c} levels in people with type 2 diabetes. Unfortunately, this study failed to achieve periodontal treatment outcomes comparable to those obtained by several existing studies among people with diabetes in several countries, as well as by studies enrolling persons without known diabetes.

We call on the periodontal community to urgently analyze why large multi-center RCTs appear incapable of effectively treating periodontitis to accepted standards of care. We also wonder why such costly studies do not specify in their protocols that periodontal treatment should be performed to defined clinical endpoints, as suggested in 2008 by Armitage[18] and in 2010 by Offenbacher and Beck.[19] Such adaptive treatment protocols would eliminate incomplete or inadequate therapeutic outcomes and their potential to mislead readers into believing that any treatment provided as "per protocol" would automatically lead to clinically significant improvements in periodontal health, which subsequently would affect the outcome studied.

Consequences

We are very concerned that, despite these inconclusive results, the outcomes of this RCT are quoted as "definitive." Despite the lack of clinically significant improvements in periodontal health, and because the authors claim there were significant improvements – without using the qualifier "statistically significant only" – the study is quoted by the press as demonstrating ("proving") that there is no effect of periodontal treatment on glycated hemoglobin. Additionally, this study did not address the degree of obesity of test subjects at all, and the title of the study may mislead the public into believing that the results are applicable to all cases of type 2 diabetes. This is an unsafe and incorrect conclusion and dangerously misleading to the profession, the public, and other stakeholders, such as policy makers, health plan managers, and insurance companies.

Given the inconclusive nature of these data, we recommend that the existing body of evidence in which meta-analyses consistently conclude that successful periodontal therapy appears to improve glycemic control, should instruct us until results from future studies are reported. We urge all interested parties to refrain from using these study results as a basis for future scientific texts, new research projects, guidelines, policies, and advice regarding the incorporation of necessary periodontal treatment in diabetes management.

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Resveratrol suppresses the inflammatory responses of human gingival epithelial cells in a SIRT1 independent manner

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Background and Objective: In periodontitis, chronic infection by periodontopathic bacteria induces uncontrolled inflammation, which leads to periodontal tissue destruction. Human gingival epithelial cells (HGECs) constitute a critical first line of defense against periodontopathic bacteria, both as a physical barrier and as regulators of inflammation. Resveratrol, a polyphenol found in grapes and red wine, reportedly has anti-inflammatory properties. Therefore, we investigated the effects of resveratrol on the *Porphyromonas gingivalis*-induced inflammatory responses of HGECs and their mechanism.

Material and Methods: We stimulated the HGEC line, epi 4, with live or heat-killed *P. gingivalis* in the presence of resveratrol, and analyzed expressions of the interleukin-8, monocyte chemoattractant protein-1 and interleukin-1β genes. We determined the involvement of SIRT1 in the effect of resveratrol using sirtinol (a SIRT1 inhibitor) or *SIRT1* knockdown. We also examined whether the effects were mediated by activation of AMP-activated kinase, suppression of reactive oxygen species, or inhibition of nuclear factor-κB (NF-κB).

Results: Resveratrol treatment decreased the expression of inflammatory cytokines and slightly increased the expression of *SIRT1*. However, neither SIRT1 inhibition nor *SIRT1* knockdown counteracted its anti-inflammatory effects. Although resveratrol did not affect AMP-activated kinase activation or reactive oxygen species production, it slightly suppressed NF-κB translocation when cells were stimulated with heat-killed *P. gingivalis*.

Conclusion: Resveratrol suppressed the inflammatory responses of *P. gingivalis*-stimulated HGECs, probably by inhibiting NF-κB signaling but independent of SIRT1.

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Key words: anti-inflammatory effect; human gingival epithelial cell; resveratrol; SIRT1

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Periodontitis is characterized by the inflammation and destruction of tooth-supporting tissues, and results from the interaction between host defense mechanisms and periodontopathic bacteria that constitute dental plaque biofilm and penetrate gingival tissue (1). Human gingival epithelial cells (HGECs) provide a critical first

line of defense against these bacteria, by not only serving as a physical barrier but also regulating the immune responses. HGECs produce proinflammatory cytokines and chemokines in response to periodontopathic bacteria or bacterial components (2). Appropriate immune responses are indispensable to combat these bacteria; however, uncontrolled inflammation caused by chronic infection leads to the destruction of periodontal tissue.

Resveratrol, a polyphenol produced by plants in response to external stressors and infections, is found in grapes and red wine in minute amounts. Resveratrol mimics the health-promoting effects of calorie restriction, and was shown to protect against aging-related diseases such as type 2 diabetes, cardiovascular diseases and cancer in tissue culture and animal models (3–5). Recently, several clinical trials have reported beneficial effects of resveratrol, particularly on cardiovascular diseases and diabetes (6). In addition, resveratrol was shown to exhibit anti-inflammatory properties in cell culture and rodent models (4,5,7,8). Some clinical trials have shown that resveratrol decreases serum inflammatory mediators, including high-sensitivity C-reactive protein and proinflammatory cytokines (9,10); however, others contradict these findings (11,12).

The molecular targets of resveratrol remain unclear. Many studies support the hypothesis that SIRT1 mediates the beneficial effects of resveratrol (13). SIRT1 is one of seven mammalian Sirtuins (SIRT1–7) – a conserved family of NAD^+-dependent deacetylases involved in numerous fundamental cellular processes, including gene silencing, DNA repair and metabolic regulation. AMP-activated protein kinase (AMPK) is also a target of resveratrol's metabolic effects (14). AMPK senses the intracellular AMP/ATP ratio and, upon a reduction in energy stores, increases ATP generation. Conversely, resveratrol exhibits antioxidant properties by scavenging reactive oxygen species (ROS) and regulating enzymes involved in oxidation (15). Moreover, resveratrol inactivates nuclear factor-κB (NF-κB), a transcription factor involved in inflammatory cytokine production and leukocyte activation, which may result from decreased SIRT1 activation or ROS production (16).

In brief, resveratrol regulates pathologic inflammation; therefore, it may be beneficial for the prevention or treatment of periodontitis. In this study, we examined the effects of resveratrol on *Porphyromonas gingivalis*-induced inflammatory responses of HGECs, and investigated their mechanism, focusing on putative molecular targets of resveratrol.

Table 1. The effect of resveratrol on the viability of epi 4 cells

Concentrations of resveratrol (μM)	Absorbance values
0 (Control)	0.285 ± 0.022
1	0.266 ± 0.013
2	0.282 ± 0.014
4	0.288 ± 0.009*
8	0.298 ± 0.004
15	0.282 ± 0.014
30	0.236 ± 0.013**
60	0.149 ± 0.005**

Absorbance values are expressed as the mean ± SD of triplicate cultures.
*$p < 0.05$; **$p < 0.01$ vs. control.

Material and methods

Reagents

Resveratrol (trans-resveratrol), sirtinol and AICAR were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies to SIRT1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Antibodies to AMPKα, phospho-AMPK (Thr172) and NF-κB p65 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Histone deacetylase 1 antibody was obtained from GeneTex, Inc. (Irvine, CA, USA).

Bacterial culture

P. gingivalis strain W83 was cultured in modified Gifu anaerobic medium

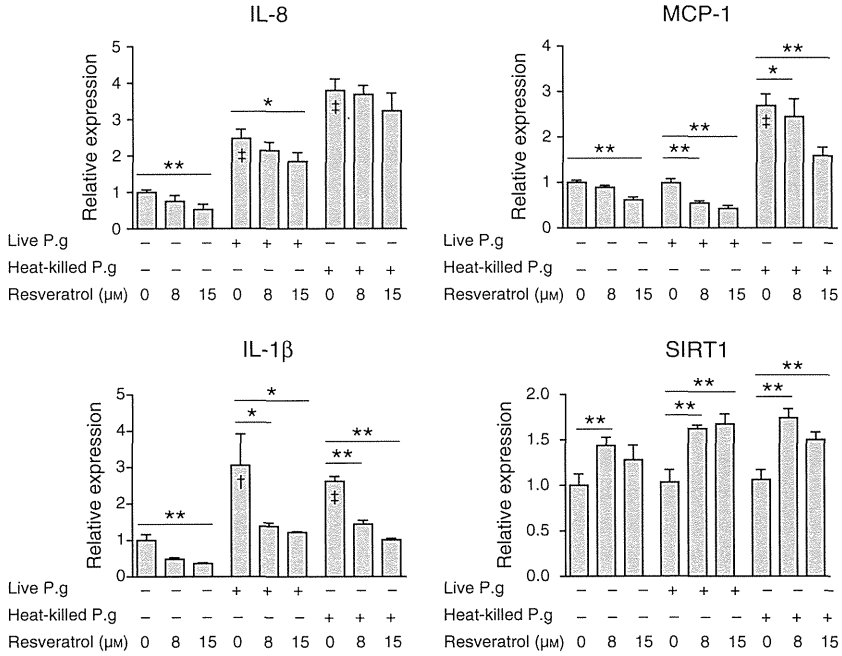

Fig. 1. The anti-inflammatory effects of resveratrol. Epi 4 cells were cultured for 6 h in the presence of 8 or 15 μM resveratrol. In the last 4 h of culture, cells were stimulated with live or heat-killed P.g, and gene expressions were analyzed using real-time polymerase chain reaction. The relative expression level of each mRNA was normalized to that of GAPDH mRNA. Data are expressed as the mean ± SD of triplicate cultures. *$p < 0.05$; **$p < 0.01$ vs. no resveratrol treatment. †$p < 0.05$; ‡$p < 0.01$ vs. no stimulation. IL, interleukin; MCP, monocyte chemoattractant protein; P.g, *P. gingivalis*.

broth (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) in an anaerobic jar (Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) in the presence of an AnaeroPack™ (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan) for 48 h at 37°C. Bacterial suspensions were prepared in phosphate-buffered saline without Mg^{2+}/Ca^{2+} using established growth curves and spectrophotometric analysis. The number of colony forming units was standardized by measuring optical density at 600 nm. Where appropriate, bacteria were heat-killed at 95°C for 10 min before addition to cell culture.

Cell culture

The SV40 T-antigen-immortalized HGEC line, epi 4, was maintained in EpiLife Medium supplemented with S7 (Life Technologies, Carlsbad, CA, USA) and antibiotics. Epi 4 cells retain the basic characteristics of primary HGECs (2,17). For stimulation experiments, epi 4 cells were seeded in a 24-well culture plate at 2×10^5 cells/well, unless otherwise stated. After 24 h of incubation, cells were extensively washed and attached cells were used. To analyze the effects of resveratrol, epi 4 cells were cultured for 6 h (24 h for the cytokine assay) in the presence of resveratrol in antibiotic-free medium. In the last 4 h (22 h for the cytokine assay) of culture, cells were stimulated with live or heat-killed *P. gingivalis* at a multiplicity of infection of 50. In some experiments, sirtinol, a SIRT1 inhibitor, was added to the culture simultaneously to the addition of resveratrol. For the analysis of NF-κB activation, epi 4 cells, in a six-well culture plate at 5×10^5 cells/well, were cultured for 3.5 h in the presence of resveratrol. In the last 1.5 h of culture, cells were stimulated with *P. gingivalis*.

Thiazolyl blue tetrazolium bromide (MTT) assay

Epi 4 cells were seeded in a 96-well culture plate at 5×10^4 cells/well and exposed to various concentrations of resveratrol for 24 h. Cells were incubated for an additional 2 h in the presence of the MTT solution (Sigma-Aldrich). Cell viability was assessed by measuring the absorbance of cell lysates at 570 nm.

Gene expression analysis

Total RNA was isolated from epi 4 cells using TRIzol Reagent (Life Technologies). cDNA was synthesized as described previously (18) and amplified using TaqMan Gene Expression Assay primer/probe sets for mRNAs (Applied Biosystems, Foster City, CA, USA) and EagleTaq Master Mix (Roche Molecular Systems Inc., Branchburg, NJ, USA), according to the manufacturer's instructions. The polymerase chain reaction was conducted using a LightCycler 96 System (Roche Diagnostics, Basel, Switzerland). The relative expression level of each mRNA was normalized to that of *GAPDH* mRNA using the $2^{-\Delta\Delta Ct}$ cycle threshold method.

Cytokine assay

The levels of interleukin (IL)-8 (Life Technologies), monocyte chemoattractant protein-1 (MCP-1) and IL-1β (R&D Systems, Inc., Minneapolis, MN, USA) in the supernatants of each culture were determined using commercially available ELISA kits.

siRNA-mediated gene knockdown

A Silencer Select siRNA against *SIRT1* and a negative control siRNA were purchased from Life Technologies. The sequence of the *SIRT1* siRNA was 5′-CGAUGUUUGAUAUU GAAUAtt-3′ (sense) and 5′-UAUUC AAUAUCAAACAUCGct-3′ (antisense). The sequence of the negative control siRNA has not been published. Cells were transfected with 5 nM siRNA using Lipofectamine RNAiMAX (Life Technologies) using the reverse transfection method in antibiotic-free medium. After 24 h of incubation, cells were washed and

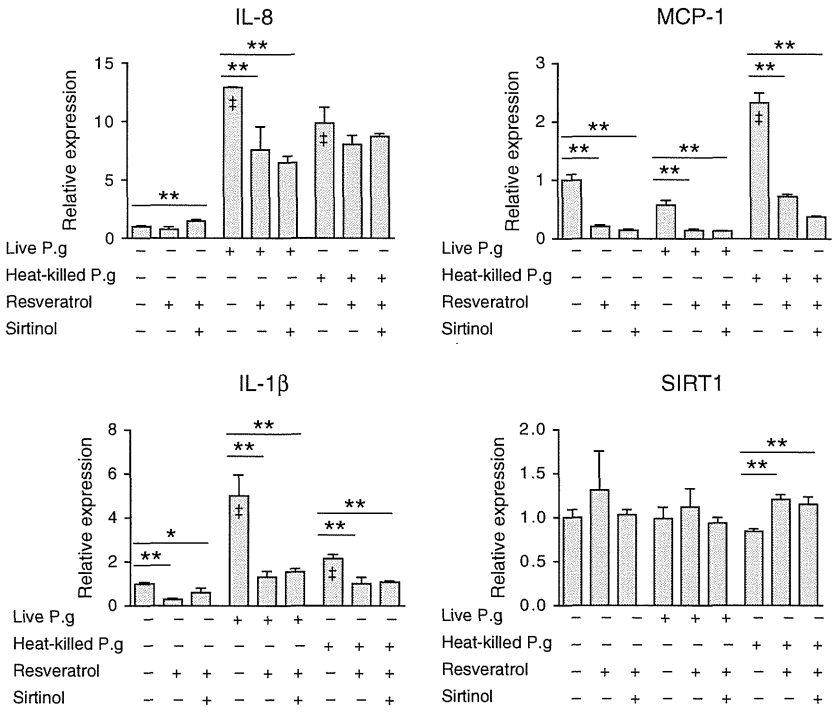

Fig. 2. The effect of SIRT1 inhibition on the effects of resveratrol. Epi 4 cells were cultured for 6 h in the presence of 15 μM resveratrol and/or 5 μM sirtinol (a SIRT1 inhibitor). In the last 4 h of culture, cells were stimulated with P.g. Gene expressions were analyzed using real-time polymerase chain reaction. Data are expressed as the mean ± SD of triplicate cultures. $*p < 0.05$; $**p < 0.01$ vs. no treatment with either resveratrol or sirtinol. $‡p < 0.01$ vs. no stimulation. IL, interleukin; MCP, monocyte chemoattractant protein; P.g, *P. gingivalis*.

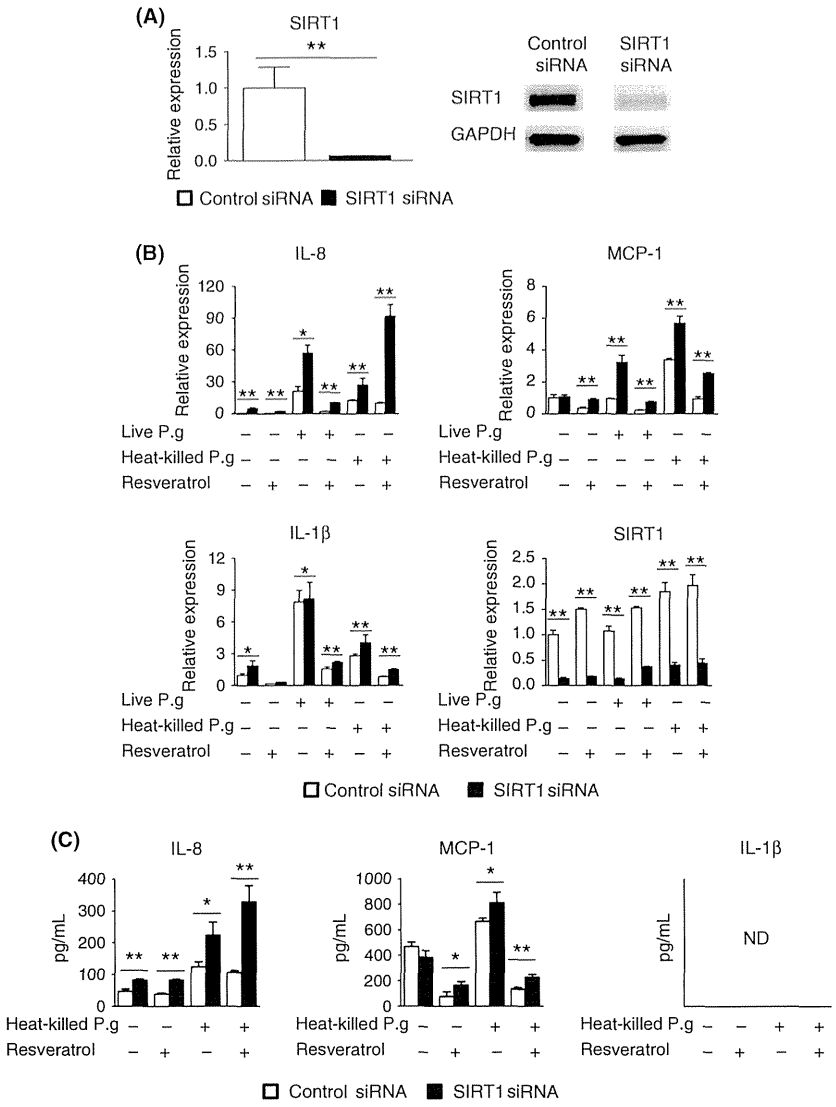

Fig. 3. The effect of SIRT1 knockdown on the effects of resveratrol. (A) SIRT1 knockdown was achieved by 24 h of siRNA transfection in epi 4 cells. The bar graph (left) indicates the expression of SIRT1 analyzed by real-time polymerase chain reaction, whereas the figures (right) show protein expressions determined by western blotting. (B,C) siRNA-transfected cells were cultured for 6 h (B) or 24 h (C) in the presence of 15 μM resveratrol. In the last 4 h (B) or 22 h (C) of culture, cells were stimulated with P.g. Gene expressions (B) or protein levels in culture supernatants (C) were measured by real-time polymerase chain reaction and enzyme-linked immunosorbent assay, respectively. Data are expressed as the mean ± SD of triplicate cultures. White and black bars indicate transfection with control and SIRT1 siRNAs, respectively. $*p < 0.05$; $**p < 0.01$ vs. control siRNA. IL, interleukin; MCP, monocyte chemoattractant protein; ND, not detected; P.g, *P. gingivalis*.

cultured for an additional 48 h before stimulation experiments.

Western blotting

Proteins were extracted from epi 4 cells using M-PER Mammalian Protein Extraction Reagent (for analysis of AMPK activation) or NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (for analysis of NF-κB activation) supplemented with Protease Inhibitor Cocktail and Halt Phosphatase Inhibitor Cocktail (all from Thermo Fisher Scientific Inc., Waltham, MA, USA). Ten micrograms of each sample were solubilized in sodium dodecyl sulfate sample buffer, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and subsequently transferred to polyvinylidene fluoride membranes (EMD Millipore Corporation, Bedford, MA, USA). The membranes were incubated with specific antibodies, and antibody bound proteins were detected using ECL Prime Western blotting detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and a LumiVision PRO 400EX system (Aisin Seiki Co., Ltd., Aichi, Japan). The intensity of each band was quantified as previously described (19).

Reactive oxygen species measurement

ROS production by epi 4 cells was determined using a Total ROS/Superoxide Detection Kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA) according to the manufacturer's protocols. Fluorescence intensity, representing ROS level, was measured using a FACScan flow cytometer and CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of ROS-positive cells among the total cells was measured when that of unstimulated cells was 5%. Pyocyanin (Enzo Life Sciences, Inc.) was used as a positive control.

Statistical analysis

All experiments were repeated at least three times with similar results. Data were analyzed by unpaired *t*-test using GRAPHPAD PRISM 5 (GraphPad Software, Inc., San Diego, CA, USA). The differences were considered significant at $p < 0.05$.

Results

The anti-inflammatory effects of resveratrol on *P. gingivalis*-stimulated epi 4 cells

Before stimulation experiments, non-cytotoxic concentrations of resveratrol were determined by MTT assay (Table 1). The absorbance value on spectroscopy significantly decreased with resveratrol concentrations of

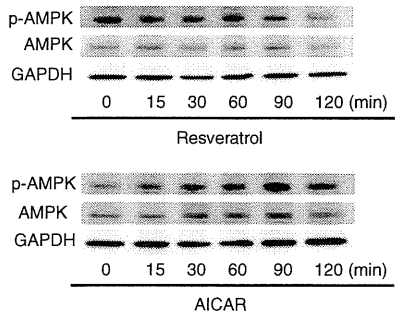

Fig. 4. Resveratrol has no effect on AMPK phosphorylation. Epi 4 cells were cultured for up to 120 min in the presence of 15 μM resveratrol or 2 mM AICAR (an AMPK activator), and total protein was extracted. The level of AMPK phosphorylation was determined by western blotting. p-AMPK, phospho-AMPK.

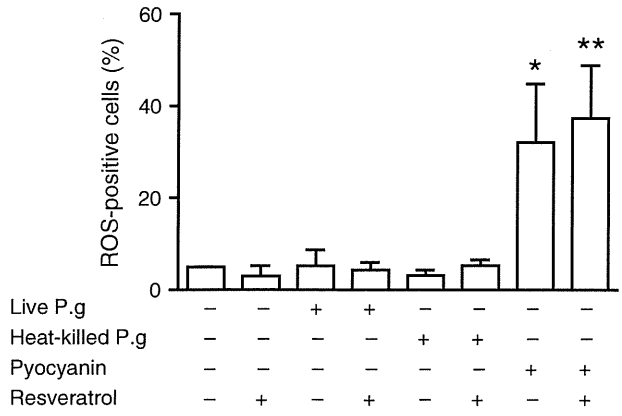

Fig. 5. Resveratrol has no effect on ROS production. Epi 4 cells were cultured for 6 h in the presence of 15 μM resveratrol. In the last 4 h of culture, cells were stimulated with P.g or pyocyanin (a ROS inducer). ROS production was detected by flow cytometry. Data show the percentage of ROS-positive cells in total cells and are expressed as the mean ± SD of three independent experiments. $*p < 0.05$; $**p < 0.01$ vs. no stimulation. P.g, *P. gingivalis*; ROS, reactive oxygen species.

30 μM or higher. Therefore, we analyzed the effect of noncytotoxic concentrations of resveratrol (8 and 15 μM) on the inflammatory responses of epi 4 cells. Stimulation of epi 4 cells with live or heat-killed *P. gingivalis* upregulated the expression of the *IL-8* and *IL-1β* genes, whereas that of *MCP-1* was increased only by stimulation with heat-killed *P. gingivalis* (Fig. 1). Treatment with resveratrol decreased the expression of the *MCP-1* and *IL-1β* genes in a dose-dependent manner, regardless of the type of stimulation. Conversely, resveratrol reduced *IL-8* expression in epi 4 cells stimulated with live but not heat-killed *P. gingivalis*. *SIRT1* expression was unchanged by stimulation with *P. gingivalis*, but its expression slightly increased in the presence of resveratrol.

The effect of SIRT1 inhibition or *SIRT1* knockdown on its anti-inflammatory properties

To elucidate the mechanism of the anti-inflammatory effects of resveratrol, we first examined the impact of sirtinol, a SIRT1 inhibitor. The concentration of sirtinol used did not affect cell viability in preliminary experiments (data not shown). Contrary to our expectations, treatment with sirtinol did not counteract the effect of resveratrol on the expression of the *IL-8*, *MCP-1* and *IL-1β* genes (Fig. 2). In addition, sirtinol barely affected the expression of *SIRT1*.

Next, we performed knockdown of *SIRT1* in epi 4 cells. A *SIRT1*-specific siRNA resulted in > 90% inhibition of *SIRT1* mRNA and a substantial decrease in SIRT1 protein (Fig. 3A). In stimulation experiments, *SIRT1* knockdown cells demonstrated increased expression of the *IL-8*, *MCP-1* and *IL-1β* genes compared with control cells (Fig. 3B). There was no apparent difference in the degree of resveratrol-induced reduction of *MCP-1* and *IL-1β* expression between *SIRT1* knockdown and control cells. Similar results were obtained regarding the expression of *IL-8* by *SIRT1* knockdown and control cells stimulated with live *P. gingivalis*. However, resveratrol further enhanced heat-killed *P. gingivalis*-induced *IL-8* expression in *SIRT1* knockdown cells. These changes in mRNA expression were confirmed by protein levels in culture supernatants after stimulation with heat-killed *P. gingivalis* alone (Fig. 3C). Live *P. gingivalis* degrades secreted proteins through proteases such as gingipain (2). The protein levels of IL-8 and MCP-1 in the supernatants reflected the changes in mRNA expression, although IL-1β was undetectable (< 2 pg/mL).

Resveratrol has no effect on AMPK phosphorylation

AICAR, an AMPK activator, increased the phosphorylation of AMPK from 30 to 120 min (Fig. 4). However, resveratrol treatment did not affect the phosphorylation of AMPK during the 120 min experimental period.

Resveratrol has no effect on reactive oxygen species production

Neither live nor heat-killed *P. gingivalis* affected ROS production, although pyocyanin, a ROS inducer, increased ROS production in epi 4 cells (Fig. 5). Resveratrol did not show any inhibitory effects on ROS production, even when ROS production was enhanced by pyocyanin.