

Inhibition of interleukin-1 β -stimulated collagenase and stromelysin expression in human tendon fibroblasts by epigallocatechin gallate ester

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Abstract

The medicinal benefits of green tea (*Camellia sinensis*) consumption have been attributed to bioavailable polyphenols, notably epigallocatechin gallate (EGCG). We have assessed the effects of EGCG and its non-esterified counterpart EGC on the expression of the collagenases, matrix metalloproteinases (MMP)-1 and -13, and the stromelysin, MMP-3, in human tendon-derived fibroblasts. Interleukin (IL)-1 β increased MMP-1, -3 and -13 mRNA and output at least 30-fold. EGCG reduced this stimulation, by 20–30% at 2.5 μ M and more than 80% at 25 μ M, and had a smaller effect on MMP-2 mRNA expression, which was not stimulated by IL-1 β . In all experiments EGCG was at least 10-fold more potent than EGC. EGCG reduced the stimulation of p54 JNK/SAPK phosphorylation by IL-1 β but did not affect p38 MAPK phosphorylation, the degradation of I κ B or the activating phosphorylation of NF κ B. We conclude that EGCG reduces the IL-1-stimulated expression of both collagenase and stromelysin mRNA species, an effect which may be mediated by inhibition of the JNK/SAPK pathway. Taken together with previous reports of EGCG effects on the expression and/or activity of gelatinases and aggrecanases, our results underline the importance of extracellular matrix breakdown as a potential target for the actions of green tea polyphenols.

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Keywords: Epigallocatechin gallate; Interleukin-1 β ; Matrix metalloproteinase; Aggrecanase; Green tea; Tendon

1. Introduction

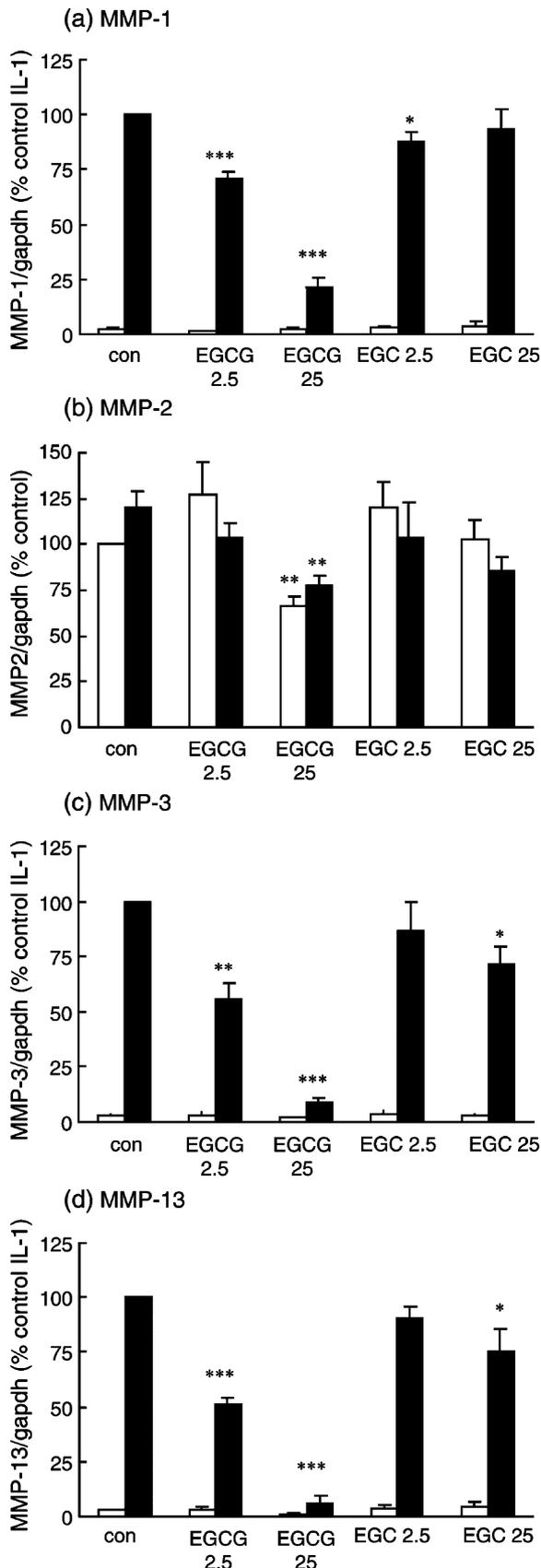
The reported benefits of green tea (*Camellia sinensis*) consumption include anti-tumour and anti-arthritis actions (Mukhtar and Ahmed, 2000; Dufresne and Farnworth, 2001). These have been attributed to a family of bioavailable polyphenols, of which epigallocatechin gallate (EGCG) is both the most abundant and one of the most potent in its antioxidant and biological activities (Mukhtar and Ahmed, 2000; Dufresne and Farnworth, 2001; Lambert and Yang, 2003); epigallocatechin (EGC), lacking the gallate ester moiety, is substantially

less active than EGCG. Several studies have indicated that the anti-tumour activity of EGCG may correlate with an inhibition of the expression, secretion and/or activation of the gelatinases, matrix metalloproteinase (MMP)-2 and MMP-9, and their activator, membrane-type (MT)1-MMP (Annabi et al., 2002; Dell'Aica et al., 2002; Maeda-Yamamoto et al., 2003). EGCG has also been shown to inhibit the breakdown of both aggrecan and collagen in a model of cartilage degradation (Adcocks et al., 2002). Aggrecan breakdown in this system is mediated by specific aggrecanases, members of the ADAMTS (A Disintegrin And Metalloproteinase with Thrombospondin motifs) group of metalloproteinases (Caterson et al., 2000; Tortorella et al., 2001). The inhibition by EGCG of aggrecan breakdown may be due to a direct inhibition of these aggrecanases, since EGCG is a selective inhibitor of ADAMTS-1, -4 and -5, with sub-micromolar IC₅₀ (Van-kemmelbeke et al., 2003).

Abbreviations: ADAMTS, A Disintegrin And Metalloproteinase with Thrombospondin motifs; Ct, threshold cycle; EGC, epigallocatechin; EGCG, epigallocatechin gallate; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; IL-1 β , Interleukin-1 β ; (MT1)-MMP, (membrane type 1)-matrix metalloproteinase.

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Much less is known about the effects of the green tea polyphenols on other MMPs, notably the collagenases, MMP-1 and -13, and the stromelysin, MMP-3. Direct inhibition of the collagenases by EGCG has been reported (Makimura et al., 1993; Vankemmelbeke et al., 2003), but this was only a partial effect, occurring at concentrations two orders of magnitude higher than those which inhibited the aggrecanases in the same study (Vankemmelbeke et al., 2003). This suggests that any inhibition by EGCG of collagen breakdown may instead be due to an effect on enzyme expression or activation. Previous evidence for effects of polyphenols on MMP-1 expression is limited to two apparently contradictory studies in human dermal fibroblasts: EGCG inhibited UV-stimulated MMP-1 mRNA expression (Kim et al., 2001), whereas EGC stimulated MMP-1 mRNA expression irrespective of UV treatment (Soriani et al., 1998). We are interested in the pathological and pharmacological modulation of matrix turnover in tendon (Riley et al., 1994, 2002; Corps et al., 2002) and have now compared the effects of EGCG and EGC on the expression of MMP-1, -2, -3 and -13 mRNA in human tendon-derived fibroblasts stimulated with interleukin (IL)-1 β .

2. Results

2.1. Effects of EGCG on expression of MMPs

Tendon-derived cells expressed each of the MMP mRNA examined. Differences between the Ct values in the optimised semi-quantitative RT-PCR assays indicated that, in unstimulated fibroblasts, MMP-2 mRNA was constitutively expressed at levels at least 100-fold higher than MMP-1 or MMP-3 mRNA (i.e. a difference of more than 7 Ct units), while the level of MMP-13 mRNA was a further 100-fold lower and close to the detection limit (data not shown). As found previously (Corps et al., 2002; Tzusaki et al., 2003), incubation of tendon cells with IL-1 β resulted in an increased expression of MMP-1 and MMP-3 mRNA (typically 100-fold and 30-fold), while MMP-2 mRNA was not substantially affected (Fig. 1a–c). The level of MMP-1 and MMP-3 mRNA in the stimulated cells was, therefore, within

Fig. 1. Effects of EGCG and EGC on MMP mRNA expression in tendon-derived cells. Cells were given control pretreatment (con) or pretreated with EGC or EGCG at 2.5 or 25 μ M for 18 h. IL-1 β (filled columns) or vehicle (open columns) was added and the incubation was continued for 24 h before isolating total RNA. MMP mRNA was normalised to GAPDH mRNA. Values for MMP-1, -3, and -13 mRNA were expressed as a percentage of those in cells treated with IL-1 β after control pretreatment. Values for MMP-2 were expressed as a percentage of those in control cells. Mean \pm S.E.M. from five independent experiments. Significant effects of EGC or EGCG treatment compared to the 100% values were determined from confidence intervals: * P < 0.05; ** P < 0.01; *** P < 0.001.

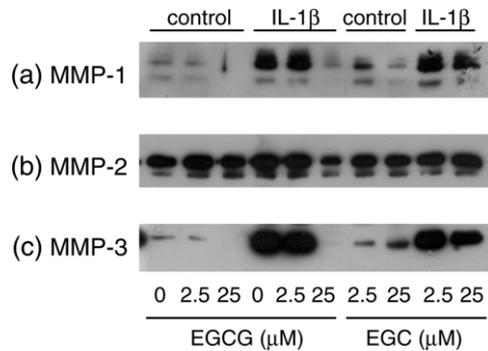


Fig. 2. Effects of EGCG and EGC on MMP secretion by tendon-derived cells. Cells (parallel incubations to those described in Fig. 1) were given control pretreatment (o) or pretreated with EGC or EGCG at 2.5 or 25 μM for 18 h. IL-1 β or vehicle was added and the incubation was continued for 48 h, and supernatant medium was analysed by Western blotting for (a) MMP-1, (b) MMP-2, (c) MMP-3. Similar results were obtained in four additional experiments.

an order of magnitude (up to 3 Ct units) of that of MMP-2 mRNA. MMP-13 mRNA also showed an increase of at least 30-fold after stimulation (Fig. 1d), but was more than 30-fold (at least 5 Ct units) lower than the others. The ease of detection of the secreted MMP proteins was consistent with these differences of mRNA levels (see below).

Incubation of the cells with EGCG gave a dose-dependent reduction in IL-1 β -stimulated MMP-1, -3 and -13 mRNA expression (Fig. 1a, c and d); typical reductions of 20–30% in stimulation were obtained at 2.5 μM EGCG, while more than 80% reduction occurred at 25 μM EGCG. In each experiment, EGC had either no effect or was less potent than EGCG and, taking the five experiments together, none of the effects of EGC was statistically significant (Fig. 1a, c and d). MMP-2 mRNA expression was reduced by 20–40% by EGCG at the higher dose irrespective of the presence of IL-1 β (Fig. 1b).

Consistent with the respective mRNA levels, both MMP-1 and MMP-3 proteins were barely detectable by Western blotting of supernatant medium from unstimulated cells but were readily detected after stimulation, while the secretion of MMP-2 was readily detected in supernatant medium from both unstimulated and IL-1 β -stimulated cells (Fig. 2). Consistent with the much lower level of MMP-13 mRNA, the level of MMP-13 secretion was below the detection level even after stimulation (and is, therefore not shown). When the cells were pretreated with 25 μM EGCG, the output of MMP-1 and MMP-3 stimulated by IL-1 β was reduced by more than 90% (Fig. 2), while any effect of EGC was substantially smaller. The output of MMP-2, from control or stimulated cells, was partly reduced by 25 μM EGCG (Fig. 2). Each of MMP-1, MMP-2 and MMP-3 were detected mainly as the proenzyme form,

with a small proportion of active MMP-1 and MMP-2; the levels of both proenzyme and active forms were reduced by EGCG.

2.2. Effects of EGCG on early signalling responses and NF κ B activation

EGCG has been reported to inhibit phosphorylation responses in signalling cascades and the activation of transcription factors (see Discussion), both of which might affect the induction of MMP expression by IL-1. As in other cell types, the addition of IL-1 β to tendon cells induced a rapid transient increase in phosphorylation of p38 MAPK and p54 JNK/SAPK, which was maximal at approximately 15 min (Fig. 3) and returned to near-basal levels by 60 min (not shown). Neither EGCG (Fig. 3a) nor EGC (not shown) affected the stimulation of p38 MAPK phosphorylation, but EGCG (25 μM) caused a reduction in the stimulated phosphorylation of p54 JNK/SAPK, with no consistent effect on the total level of p54 JNK/SAPK (Fig. 3b). The reduction in the stimulated phosphorylation of p54 JNK/SAPK was estimated, by scanning the blots, to be between 50 and 75%. The phosphorylation of p42/p44 ERK was neither greatly stimulated by IL-1 β nor reduced by EGCG in these cells (not shown).

The inhibitory regulator I κ B, which retains the transcription factor NF κ B inactive in the cytoplasm, decreased to almost undetectable levels after 15 min incubation with IL-1 β and began to increase again by 60 min (Fig. 4a). Neither EGCG (Fig. 4a) nor EGC (not shown) affected either the decrease of I κ B or its subsequent re-appearance. In the same samples, the phosphorylation of NF κ B was reversibly stimulated by IL-1 β , and this was not affected by either EGCG (Fig. 4b) or EGC (not shown).

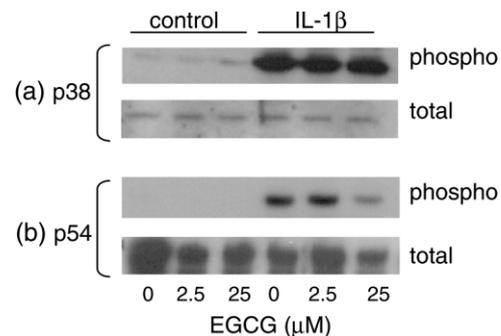


Fig. 3. Effects of EGCG on signalling responses in tendon-derived cells. Cells were given control pretreatment (o) or pretreated with EGCG at 2.5 or 25 μM for 18 h. IL-1 β or vehicle was added and the incubation was continued for 15 min. Cell extracts were analysed by Western blotting for (a) phosphorylated and total p38 MAPK, and (b) phosphorylated and total p54 JNK/SAPK, as indicated. Similar results were obtained in two additional experiments.

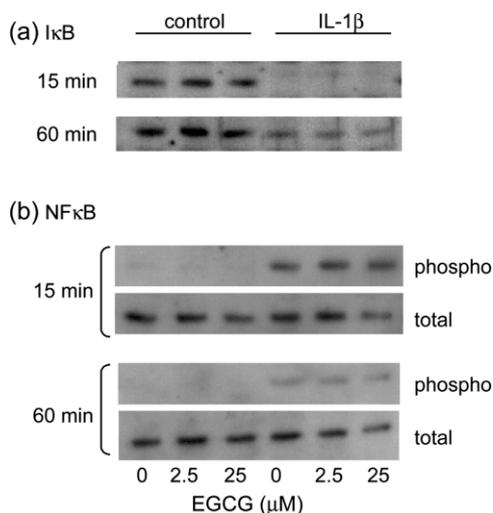


Fig. 4. Effects of EGCG on NFκB activation in tendon-derived cells. Cells were given control pretreatment (o) or pretreated with EGCG at 2.5 or 25 μM for 18 h. IL-1β or vehicle was added and the incubation was continued for 15 or 60 min. Cell extracts were analysed by Western blotting for (a) IκB, and (b) phosphorylated and total NFκB, as indicated. Similar results were obtained in two additional experiments.

3. Discussion

The beneficial effects of green tea consumption may involve actions of EGCG against matrix breakdown, by a combination of effects on the expression and activity of metalloproteinases. Previous studies have focussed on the effects of EGCG on the expression of the gelatinase MMP-2 and its activation by MT1-MMP in cancer cells (Annabi et al., 2002; Dell'Aica et al., 2002; Maeda-Yamamoto et al., 2003), including effects on the expression of these genes (Annabi et al., 2002; Maeda-Yamamoto et al., 2003). We have found that, at the higher concentration tested, EGCG also reduced the expression of MMP-2 in cultured tendon cells. However, the principal novel finding of the present study is that EGCG substantially reduced IL-1β-stimulated expression of the collagenases MMP-1 and MMP-13 and the stromelysin MMP-3, through an inhibition of stimulated mRNA expression (Figs. 1 and 2). EGC was substantially less potent than EGCG, consistent with their relative potency in many other systems (Mukhtar and Ahmed, 2000; Dufresne and Farnworth, 2001).

The inhibitory effects of EGCG occurred over the range 2.5–25 μM, which is similar to that used in studies of various effects of EGCG on cells in vitro (Dong et al., 1997; Nam et al., 2001; Annabi et al., 2002; Lambert and Yang, 2003). The reported levels of EGCG found in blood after ingestion of green tea range up to 2 μM (Yang et al., 1998) or 5 μM (Scalbert and Williamson, 2000). The significant effects of 2.5 μM EGCG on the expression of MMP mRNA (typically

20–30%) are, therefore, pharmacologically relevant, and it is also possible that higher levels of EGCG might accumulate in the tissue. The cumulative effects over time of partial reduction of MMP expression in vivo are not known.

The inhibition of MMP-1 and MMP-13 expression occurred at lower EGCG concentrations than those required for partial inhibition of collagenase activity (Makimura et al., 1993; Vankemmelbeke et al., 2003). This indicates that inhibition of collagenase expression rather than direct inhibition of enzyme activity is likely to be a more significant effect of EGCG in preventing tissue collagen breakdown, e.g. in cartilage (Adcocks et al., 2002). By contrast, inhibition by EGCG of tissue aggrecan breakdown may result principally from the direct inhibition of aggrecanase (ADAMTS) activity, which occurred at sub-micromolar EGCG concentrations (Vankemmelbeke et al., 2003) and is, therefore, among the more potent of reported responses to EGCG in vitro (see review by Lambert and Yang, 2003). Rather higher concentrations of EGCG (similar to those affecting MMP expression) were required for any effect on ADAMTS-1, -4 and -5 mRNA expression in tendon cells or chondrocytes (ANC, unpublished data). Although MMP-3 may contribute to proteoglycan breakdown, there are many other potential targets of MMP-3 activity (Cawston, 1998), such that inhibition of MMP-3 expression by EGCG may have significantly wider-ranging effects.

The regulation of MMP mRNA expression by growth factors and cytokines (including IL-1β involves distinct MAPK pathways leading to the activation of transcription factors including AP-1 and NFκB, albeit with some differences between the different MMP genes and in different cell-types (Borden and Heller, 1997; Bond et al., 1999; Mengshol et al., 2000; Liacini et al., 2002; Reunanen et al., 2002). Contrasting inhibitory effects of EGCG on particular MAPK pathways have been reported (Dong et al., 1997; Maeda-Yamamoto et al., 2003; Singh et al., 2003). In the present experiments, EGCG had no effect on p38 MAPK or p42/44 ERK phosphorylation, but reduced the stimulation of p54 JNK/SAPK phosphorylation (Fig. 3); similar results have been reported in other cell types (Dong et al., 1997; Singh et al., 2003). The effect on p54 JNK/SAPK activation would be expected to reduce AP-1 activity, as found in tumor-promoter-stimulated epidermal cells (Dong et al., 1997). Taken together with previous studies indicating the involvement of AP-1 in stimulated MMP expression (Borden and Heller, 1997; Mengshol et al., 2000; Liacini et al., 2002), our results indicate that inhibition of the JNK/SAPK pathway may at least partly account for the inhibition by EGCG of MMP expression.

The transcription factor NFκB has also been shown to be critically involved in the regulation of MMP-1, MMP-3 and MMP-13 expression, in some cell types but

not others (Bond et al., 1999; Mengshol et al., 2000). Activation of NF κ B involves its release from the inhibitory factor I κ B (which is phosphorylated by I κ B kinase and targeted for destruction at the proteasome), followed by the phosphorylation of the released NF κ B. EGCG has been reported to inhibit both the I κ B kinase complex (Yang et al., 2001) and proteasome function (Nam et al., 2001), thus preventing I κ B degradation and NF κ B activation (Kim et al., 2001; Nam et al., 2001; Yang et al., 2001; Singh et al., 2002). However, although IL-1 β induced the expected disappearance of I κ B in tendon cells and the phosphorylation of NF κ B, EGCG did not inhibit either of these effects (Fig. 4). The difference between this and the earlier reports may reflect the cell-type (normal fibroblasts were more resistant than their transformed counterparts to effects of EGCG on the proteasome: Nam et al., 2001), or the precise conditions used, notably the EGCG concentration or incubation time. The results presented here were obtained using an overnight preincubation with EGCG to ensure equilibration with intracellular targets. However, similar results were obtained in two further experiments in which EGCG was added together with IL-1 β : again, EGCG inhibited IL-1 β -stimulated MMP expression, but did not inhibit NF κ B phosphorylation (data not shown). Our results therefore indicate that, at least in tendon cells, EGCG can inhibit MMP expression under conditions where it does not inhibit the activation of NF κ B.

While this paper was in preparation, Ahmed et al. (2004) reported the inhibition by EGCG of IL-1 β -induced MMP-1 and MMP-13 in human chondrocytes. In these cells, EGCG (20 μ M) had significantly greater inhibitory effects on the expression of MMP-13 than on that of MMP-1, and reduced NF κ B activity more markedly than that of AP-1. Hence, although tendon cells and chondrocytes both show inhibition of MMP expression by EGCG, the two studies differ in both the details of the inhibition and the pathways involved, supporting the suggestion (above) that there may be cell-type-specific differences in the actions of EGCG.

While the inhibition of matrix breakdown may be beneficial in degenerative diseases such as osteoarthritis, or in cancer where local degradation of the matrix may enable tumour metastasis or angiogenesis, it may be disadvantageous elsewhere. For example, in tendon there is histopathological, biochemical and molecular evidence for a balance between the synthesis and breakdown of matrix components, which is disrupted in patients suffering chronic tendinopathy (Kannus and Jozsa, 1991; Riley et al., 1994, 2002); changes in MMP-3 expression and activity have been associated with tendon degeneration and rupture (Ireland et al., 2001; Riley et al., 2002). Furthermore, tendon problems have been experienced both by patients treated with broad-spectrum metalloproteinase inhibitors (Drummond et al., 1999), and by a proportion of patients treated with

fluoroquinolone antibiotics (Pierfitte and Royer, 1996), which have been shown to increase the expression of metalloproteinases in tendon cells *in vitro* (Williams et al., 2000; Corps et al., 2002). We are not aware that tendon problems have been reported as a side-effect of green tea, but it will be of interest to observe whether there is an increased incidence of any such problems with its more widespread use.

4. Materials and methods

4.1. Materials

Dulbecco's modified Eagle's medium, fetal calf serum (FCS) and antibiotics were obtained from Invitrogen (Paisley, UK). EGC and EGCG were obtained from Sigma (Poole, UK), and were freshly dissolved at 10 mM in water. IL-1 β was a gift from Glaxo Wellcome (Stevenage, UK) and aliquots (1 μ g/ml) were stored at -70 °C. The anti-MMP-1 antibody was a rabbit polyclonal antibody (Clark et al., 1992). Antibodies against signalling kinases, NF κ B and I κ B were obtained from Cell Signalling Technology (New England Biolabs, Hitchin, UK). Other primary and secondary antibodies were from TCS Biologicals (Buckingham, UK) and Dako (Ely, UK), and CDP-Star detection reagent was from Roche Diagnostics (Lewes, UK).

4.2. Cell isolation and incubation

Tendon specimens were obtained from tissue discarded during surgery for chronic Achilles tendinopathy, with informed patient consent and local ethical committee approval. Cells isolated by outgrowth from four separate tendon explants were maintained and passaged in DMEM containing 10% (v/v) FCS, penicillin, streptomycin and 25 mM HEPES, and were used between passages 4 and 10. Cells were seeded at 10^5 /well in 6-well plates and were incubated for 3 days before the experiment. They were rinsed with serum-free medium containing insulin, transferrin and selenium, and were incubated in 2 ml of the same medium with or without EGCG or EGC for 18 h, before adding IL-1 β (1 ng/ml: approx 60 pM). Control cultures received equivalent additions of medium. The cells were then incubated for a further 24 h or 48 h, after which the medium was removed and stored at -20 °C, and the cells were rinsed with balanced salts solution and solubilised in TRI-Reagent (Sigma; 1 ml/well). Parallel plates were incubated for 15 or 60 min after the addition of IL-1 β , then placed on ice, rinsed with ice-cold balanced salts solution and lysed in extraction buffer (10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na₃VO₄, 50 mM NaF, 10 mM Na₄P₂O₇, 1 mM PMSF, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, 1% NP-40) for analysis of signalling proteins.

4.3. RNA isolation and analysis by RT-PCR

RNA was isolated from the TRI-Reagent extracts by phenol-chloroform phase separation followed by precipitation with isopropanol and ethanol. RNA was checked by agarose gel electrophoresis, showing no evidence of breakdown and no gross differences in relative levels that would account for the changes observed with different treatments.

RNA was assayed for GAPDH, MMP-1, -2, -3 and -13 mRNA, using semi-quantitative RT-PCR in a GeneAmp 5700 (Applied Biosystems, Warrington, UK). Oligonucleotide primers were obtained from Invitrogen. One-Step RT-PCR reagents and FAM-labelled oligonucleotide probes were obtained from Applied Biosystems. The primers and probes for GAPDH, MMP-1, -2, and -3 have been described previously (Ireland et al., 2001). The forward primer (F), reverse primer (R) and probe (P) sequences for MMP-13 mRNA (amplicon 81 bp, consisting of bp 916–996 from accession number NM_002427) were designed using Primer Express (Applied Biosystems) as follows:

F = CCGAGGAGAAACAATGATCTTT
 R = GTTAAAAACAGCTCCGCATCAACCT
 P = AGATTCTTCTGGCGCCTGCATCCTC

Each amplicon was chosen to include an intron splice junction to prevent amplification of genomic DNA, and BLASTn searches (www.ncbi.nlm.nih.gov/BLAST) revealed no significant similarity to other sequences. No signal was produced if either the RNA or the reverse transcriptase step was omitted, and each primer pair generated a single product of the appropriate size.

Standard curves were run in each assay, using freshly-diluted aliquots of pooled tendon cell RNA. For each target, this produced a linear plot of threshold cycle (Ct) against log(dilution), whose slope was within 10% of the expected value, indicating a similar, near-maximum efficiency for each target. For each target, all RNA samples from an experiment were assayed in duplicate on the same plate. The values obtained for MMP mRNA expression were normalised for GAPDH mRNA expression in the same sample. Consistent with the gel analysis of the RNA, no treatments showed differences in GAPDH mRNA expression that were greater than $\pm 40\%$ from the control cells.

4.4. Western blotting

Western blotting of MMP-1, -2 and -3 in cell culture supernatants was performed as described previously (Corps et al., 2002). Western blotting of signalling kinases, $\text{I}\kappa\text{B}$ and NF κB in cell extracts was performed using the primary antibodies at 1:1000, followed by detection using alkaline phosphatase-coupled goat anti-

rabbit antibody and CDP-Star reagent (all according to the suppliers' recommendations).

Acknowledgments

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