

## THE POLYACETYLENES FALCARINOL AND FALCARINDIOL AFFECT STRESS RESPONSES IN MYOTUBE CULTURES IN A BIPHASIC MANNER

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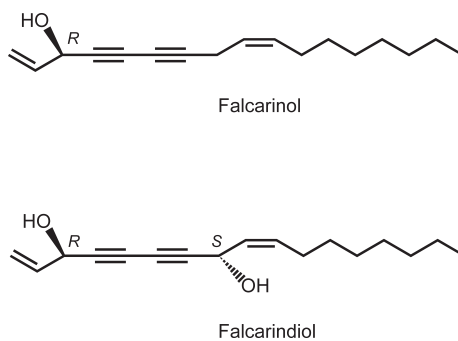
□ The effects of the bioactive polyacetylenes, falcarinol and falcarindiol, present in carrots, celery, celeriac and other umbelliferous vegetables, on the stress responses in primary myotube cultures, were studied. Biphasic responses on cellular stress responses in myotube cultures were investigated by exposing them to various concentrations of falcarinol and falcarindiol for 24 h before testing effects of 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  on the intracellular formation of reactive oxygen species (ROS), transcription of the antioxidative enzyme cytosolic glutathione peroxidase (cGPx), and the heat shock proteins (HSP) HSP70 and HO1. At low concentrations (1.6 to 25  $\mu\text{M}$ ) polyacetylenes caused a slightly accelerated intracellular ROS formation, increased cGPx transcription and decreased HSP70 and HO1 transcription. The increased cGPx transcription may be interpreted as an adaptive response to the increased ROS formation and may have caused a reduced demand for the protective functions of the HSPs. ROS formation, however, was substantially decreased after pre-incubation with both polyacetylenes at 50 and 100  $\mu\text{M}$ , the cGPx transcription was reduced and the HSP70 and HO1 transcription increased, indicating a need for the protective and repairing functions of the HSPs. In conclusion, pre-incubation with low concentrations of both polyacetylenes prior to  $\text{H}_2\text{O}_2$  exposure induced a cytoprotective effect whereas higher concentrations had adverse effects.

*Keywords:* falcarinol, falcarindiol, polyacetylenes, stress response, primary myotubes, biphasic

### INTRODUCTION

Epidemiological studies indicate some correlation between high intake of fruit and vegetables and certain beneficial health effects (Steinmetz and Potter 1991; Block et al. 1992; Maynard et al. 2003). This has led to a great interest in studying effects of single compounds originating from fruit and vegetables, e.g. bioactive compounds such as carotenoids (The Alpha-Tocopherol B-CCPSG 1994) and flavonoids (Duthie and Dobson 1999). Other highly bioactive, but less abundant compounds in fruit and/or vegetables that may contribute to overall effects, include

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**FIGURE 1.** Chemical structures of the polyacetylenes falcarinol [(3*R*)-heptadeca-1,9(*Z*)-diene-4,6-diyne-3-ol] and falcarindiol [(3*R*, 8*S*)-heptadeca-1,9(*Z*)-diene-4,6-diyne-3,8-diol] isolated from carrots.

the aliphatic C<sub>17</sub>-polyacetylenes falcarinol and falcarindiol (Figure 1), which are mainly present in carrots, celery, celeriac and other umbelliferous vegetables (Zidorn et al. 2005). Falcarinol is bioavailable in humans (Christensen and Brandt 2006) and the C<sub>17</sub>-polyacetylenes of the falcarinol type has shown biological characteristics such as anti-inflammatory (Alanko et al. 1994; Liu et al. 1998), immune stimulatory (Hansen et al. 1986), anti-platelet-aggregatory effects (Teng et al. 1989) as well as cytotoxicity (Bernart et al. 1996; Zidorn et al. 2005). In the present study we investigated the effects of falcarinol and falcarindiol on the stress responses in primary myotube cultures isolated from porcine *semimembranosus* muscle. The muscle cell was chosen as a model because it is the most abundant cell type in the human body constituting 40% of the body weight. Also this cell type is a good model for studying oxidative stress responses since oxidative stress is regularly induced in muscle cells under physical exercise. In a pilot study we have investigated stress conditions that trigger a moderate and reversible cellular stress response, as determined by HSP70 and HO1 m-RNA expression, and we found that exposure to 100 μM H<sub>2</sub>O<sub>2</sub> for 1 hour induced a background level of stress from which both decreasing and accelerating stress effects may be determined.

## MATERIALS AND METHODS

### Isolation of Falcarinol and Falcarindiol

Four kilograms of carrot roots (cv. Bolero) were ground and extracted with 5 l ethyl acetate (EtOAc) for 24 h, at room temperature in the dark. The EtOAc phase was collected by decanting, and the carrots re-extracted with another 5 l EtOAc for 24 h. The combined extracts were filtered, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated *in vacuo* (35 °C) under dim light. The extract (12 g) was chromatographed on silica gel, eluting with *n*-hexane, *n*-hexane–EtOAc (v/v) (9:1, 4:1, 7:3, 3:2, 1:1, 2:3,

1:4), and finally EtOAc (Kidmose et al. 2004). Fractions containing crude falcarinol and falcarindiol, respectively, were combined, and the individual polyacetylenes further purified by preparative reversed phase (RP) high-performance liquid chromatography (HPLC) on a Dionex Summit Preparative HPLC system (Dionex Denmark A/S, Rødovre, Denmark) controlled by Chromeleon (version 6.50) software, and equipped with a HPLC pump (P680), solvent rack (SOR-100), and a diode array detector (UV D340U) operating from 200–595 nm. Separations of polyacetylenes were performed on a Develosil ODS-HG-5 HPLC column (RP-18, 250  $\leftrightarrow$  20 mm i.d., Nomura Chemical Co., Seto, Japan), at 25 °C, using the following stepwise gradient: CH<sub>3</sub>OH–H<sub>2</sub>O [0 min (20:80), 50–60 min (100:0), 70–80 min (20:80)], yielding 45 mg falcarinol and 60 mg falcarindiol, respectively. Detection wavelength: 203 nm. Flow rate: 5 ml/min. Injection volume: 25 ml. Acquisition off at 70 min. Purity of polyacetylenes were > 96%, as determined by analytical RP-HPLC (Christensen and Kreutzmann 2007). Falcarinol and falcarindiol was obtained as colorless oils and identified by optical rotation, UV, mass spectrometry (MS) [gas chromatography (GC)–MS (EI, 70 eV)], one-dimensional and two dimensional nuclear resonance spectroscopy (NMR) [<sup>1</sup>H- and <sup>13</sup>C-NMR, <sup>1</sup>H–<sup>1</sup>H- and <sup>1</sup>H–<sup>13</sup>C-COSY] and the complete spectral data set corresponded fully with literature values (Lemmich 1981; Czepa and Hofmann 2003; Kobæk-Larsen et al. 2005).

### **Porcine primary myoblast and myotube cultures**

Myotube cultures were derived from porcine primary satellite cells isolated from *M. semimembranosus* of female pigs at an age of six weeks. The original method of Bischoff was used with some modifications (Bischoff 1974; Theil et al. 2006). Muscle tissue was excised, stripped for visible fat and connective tissue, placed in ice-cold transport medium (1% glucose, 500 IU/ml of penicillin, 500 µg/ml of streptomycin sulfate, 15 µg/ml of amphotericin and 100 µg/ml of gentamycin in Ca<sup>2+</sup> and Mg<sup>2+</sup> free phosphate buffered saline (PBS)), and transferred to a laminar flow bench. The muscle tissue was finely chopped with a pair of scissors and digested for 20 min, in 20 ml PBS (Ca<sup>2+</sup> free) containing 1% glucose, 1.5 mg/ml collagenase II, 0.25% trypsin and 0.01% DNase. As much as possible of the digestion medium was aspirated and another 20 ml digestion medium was added and left to digest for another 20 min. This procedure was repeated to give a total of 3 x 20 min digestion and a total volume of approximately 60 ml digest. Following digestion the cells were transferred to a Primary Growth Medium (PGM, DMEM (Dulbecco's modified Eagle's medium, Life Technologies, Naperville, IL) with 10% foetal calf serum (FCS, Life Technologies, Naperville, IL) and 10% horse serum (HS) supplemented with antibiotics: 100 IU/ml penicillin and 100 µg/ml streptomycin sulfate, 3 µg/ml amphotericin B, 20 µg/ml gentamycin), triturated

10 times (stripette, Costar, Cat.no. 4101, VWR, Aarhus Denmark), centrifuged at 630 x g for 8 min at 4°C, resuspended, and filtered through a 200 µm and then a 50 µm Nytex filter. Percoll gradients (20% Percoll) were used to enrich the relative proportion of satellite cells in the cell suspension (Ortenblad et al. 2003). Cells were kept in liquid nitrogen until use, where cells were thawed at 37°C and equally distributed into 24 (~ 60.000 cells/cm<sup>2</sup>) or 96-well (~ 90.000 cells/cm<sup>2</sup>) plates, coated with matrigel (1:50 v/v) and grown in PGM (95% air and 5% CO<sub>2</sub> at 37°C). For cell viability experiments cells were grown approximately 3 days to 80% of confluence before assaying, but for analysis of mRNA expression and DCFH<sub>2</sub> (2',7' dichlorodihydrofluorescein) oxidation cells were fused into myotubes prior to the experiment. Cells were made to fuse after approximately 4 days of proliferation by switching to DMEM with 10% FCS, 1 µM insulin and antibiotics for 24 h, and then to DMEM containing 5% FCS, 1 µM insulin, antibiotics and 1 µM cytosine arabinosid (fusion medium) for 72 h. The latter medium was changed after 48 h of incubation. Cells were fully differentiated after approximately 3 days in fusion medium.

#### **Experimental setup, RNA extraction and real time RT-PCR analyses**

Differentiated myotubes in 24 well plates were exposed to various concentrations (6.25-50 µM) of falcarinol or falcarindiol in the fusion medium. After 24 h fusion medium including falcarinol or falcarindiol was aspirated and the cells were washed with 1 ml/well KCl buffer (150 mM KCl, 1.3 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub> and 10 mM Hepes) before exposure to 100 µM H<sub>2</sub>O<sub>2</sub> in KCl buffer for 1 h at 37°C in 95% air and 5% CO<sub>2</sub>. KCl buffer including H<sub>2</sub>O<sub>2</sub> was aspirated, cells washed and left at 37°C in 95% air and 5% CO<sub>2</sub> in fusion medium for 18 h. Cells were washed twice in PBS, harvested in 0.25% trypsin, and stored at -80°C until extraction. RNA was extracted from the cells using the RNeasy mini kit (Qiagen, Albertslund, Denmark) and reverse transcribed with oligo-dT primers and Superscript II RNase H reverse transcriptase kit (Invitrogen, Taastrup, Denmark). Reverse Transcribed material (1 µl) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden). Primers and probe were designed specifically for each gene by using Primer Express 2.0 software (Applied Biosystems, Stockholm, Sweden) and either forward primer, minor groove binding (MGB) probe or reverse primer was designed to anneal to an exon boundary. Exon structures reported for humans or mouse (HO1) were used. Details of primer/probe design and runs of real time RT-PCR are given in Table 1. Amplicon length was tested after real time RT-PCR analysis on a 2% agarose gel and only one PCR product was amplified per gene and the amplicon length agreed with the predicted length based on the nucleotide sequences (data not shown). Quantity of mRNA was detected by gene specific MGB or TAMRA probes labelled with FAM<sup>TM</sup> fluo-

**TABLE 1.** Accession numbers, amplicon location, amplicon length, range of Ct values in samples and slope of standard curves of the analyzed genes.

Gene	Accession No.	Amplicon location (exon-exon)	Amplicon Length (bp)	Range of Ct in samples		Slope of std. curve
				Falcarinol	Falcarindiol	
HSP70	M69100	7–8	86	22.7–26.7	25.7–28.8	–3.43
HO1	AC091316	2–3	77	24.1–27.3	24.7–29.1	–3.26
cGPx	AF532927	1–1	76	25.5–28.5	24.5–27.4	–3.41
GAPDH	AF017079	2–3	76	23.8–25.7	23.5–25.4	–3.33

rophore in the 5' end and a non-flourescent quencher in the 3' end. For PCR, 40 cycles at 95°C for 15 s and 60°C for 60 s were applied to amplify the PCR products. A selected sample was diluted serially and analyzed in triplicate to test linearity and efficiency of the PCR amplifications. Furthermore, control wells with either water or genomic DNA was used as negative controls. All samples were analyzed in duplicates using the ABI 7900HT sequence detection system (Applied Biosystems, Stockholm, Sweden). The sequences of forward primers, MGB probes and reverse primers were as follows:

*Heat shock protein 70 (HSP70)*: 5'-GGCAAGGCCAACAAGATCAC-3', 5'-ACAAGGGCCGCCTGAGCAAGG-3', 5'-TTCTCAGCCTCCTGCACCAT-3'

*Heme oxygenase 1 (HO1)*: 5'-GCTGAGAATGCCGAGTTCATG-3', 5'-CAGAAGGGCGAGGTCACCCGAGA-3', 5'-GACGCCATCACCAGCTTAAAG-3'

*Glutathion peroxidase (cGPx)*: 5'-CAAGGTGCTGCTCATTGAGAAC-3', 5'-AGCATCGCTCTGAGGCACAACGGT-3', 5'-CAGGTCATTTCATCTGGGTGTAGTC-3'

*Glyceraldehyde 3 phosphate dehydrogenase (GAPDH)*: 5'- GTCGGAGTGAACGGATTTGG -3', 5'- CGCCTGGTCACCAGGGCTGCT -3', 5'- CAATGTCCACTTTGCCAGAGTTAA -3'

To evaluate mRNA quantities, data was obtained as Ct values (the cycle number at which logarithmic plots cross a calculated threshold line) according to the manufacturer's guidelines, and used to determine  $\Delta\text{Ct}$  values ( $\Delta\text{Ct} = \text{Ct of the target gene} - \text{Ct of the housekeeping gene (GAPDH)}$ ). To exclude potential bias because of averaging data that had been transformed through the equation  $2^{-\Delta\text{Ct}}$ , all statistics were performed at the  $\Delta\text{Ct}$  values. The quantitative expression of target genes was expressed relative to the level observed for cells without polyacetylene addition by calculating the  $\Delta\Delta\text{Ct}$  values ( $\Delta\text{Ct observed at a given polyacetylene concentration} - \Delta\text{Ct observed for cells without polyacetylene addition}$ ) and by using the formula: Relative quantity =  $2^{-\Delta\Delta\text{Ct}}$ . Within each compound every concentration was added to 4 wells ( $n = 4$ ) from which RNA was isolated and each isolate was analyzed for mRNA expression in duplicate.

### **Experimental setup and DCFH<sub>2</sub> oxidation analyses (ROS formation)**

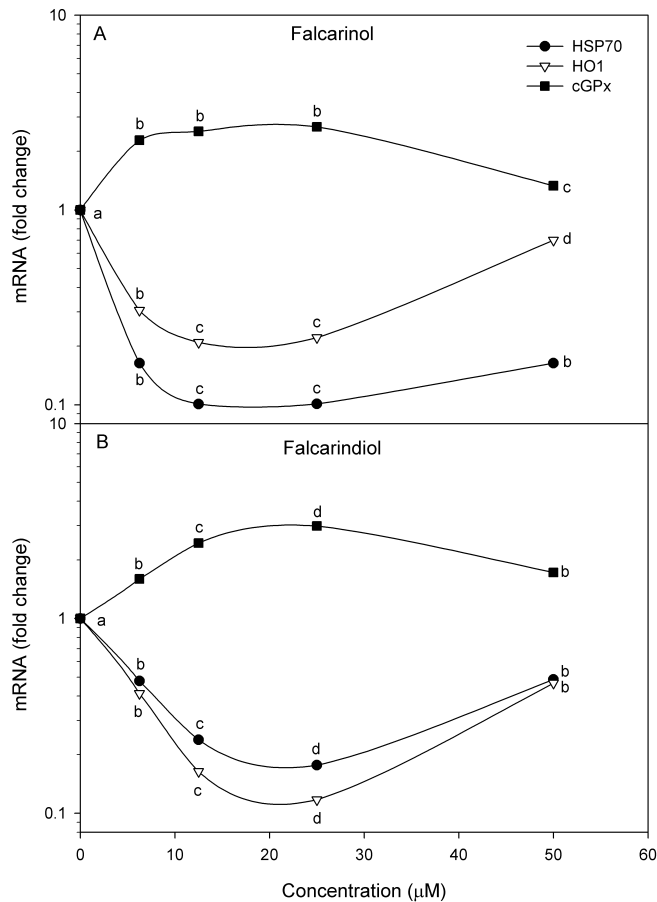
Differentiated myotubes in 96 well plates were exposed to various concentrations (1.6-100  $\mu\text{M}$ ) of falcarinol or falcarindiol in PGM for 18 h. Myotubes were washed twice in 200  $\mu\text{L}$ /well Krebs-Hepes buffer (KHB) (118 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 4.2 mM  $\text{NaHCO}_3$ , 1.3 mM  $\text{CaCl}_2$ , 1.2 mM  $\text{MgSO}_4$ , 10 mM Hepes, and 10 mM D-glucose) before they were loaded with  $\text{H}_2\text{DCF-DA}$  (10  $\mu\text{M}$ ) (2',7' dichlorodihydrofluorescein diacetate, Molecular Probes, Inc. Eugene, OR) in KHB for 2 h at 37 °C (95% air, 5%  $\text{CO}_2$ ). Buffer was aspirated and myotubes were washed twice with 200  $\mu\text{L}$ /well to remove excess extracellular  $\text{H}_2\text{DCF-DA}$ .  $\text{H}_2\text{O}_2$  (100  $\mu\text{M}$  in KCl buffer) was added to the cells and the intracellular DCFH<sub>2</sub> oxidation was determined every 4 min directly in the culture plate by fluorescence from 2,7-dichlorofluorescein (DCF) at excitation and emission wavelengths of 490 and 515 nm, respectively, at 34 °C with a microtiter plate reader (Perkin-Elmer LS50B fluorometer, Beaconsfield, U.K.), and a custom-made thermostating element from Mikrolab, (Aarhus, Denmark) for approximately 6 h. Data were corrected for background signal from wells without cells but otherwise treated similarly. All concentrations tested of falcarinol and falcarindiol were determined in triplicate wells ( $n = 3$ ).

### **Experimental setup and analyses of myoblast viability**

Viability of porcine primary myoblasts was evaluated in 96 well plates by WST-1 (Roche, Hvidovre, Denmark) a formazan salt which is cleaved by mitochondrial dehydrogenase of viable cells. The relative amount of viable cells was determined by incubating the cells with 10  $\mu\text{L}$ /well WST-1 for 4 h and measuring the absorbance at 450 nm (Oksbjerg et al. 2000). Data was corrected for the absorbance at 630 nm and background absorbance of the medium alone. All concentrations tested of falcarinol and falcarindiol were determined in quadruplicate wells ( $n = 4$ ).

### **Statistics**

The data from the experiment was analyzed by the MIXED procedure in SAS (SAS Institute, Cary, NC, USA). The absorption measured after WST-1 addition (indicating cell viability) and expression of the heat shock proteins were analyzed in a model with the concentration of falcarinol/falcarindiol as a fixed effect and replication by falcarinol/falcarindiol as a random effect. Although the ROS production was measured at several time points only one time point was analyzed statistically according to the model given for the other traits. Data is presented as LSMMeans  $\pm$  SEM.

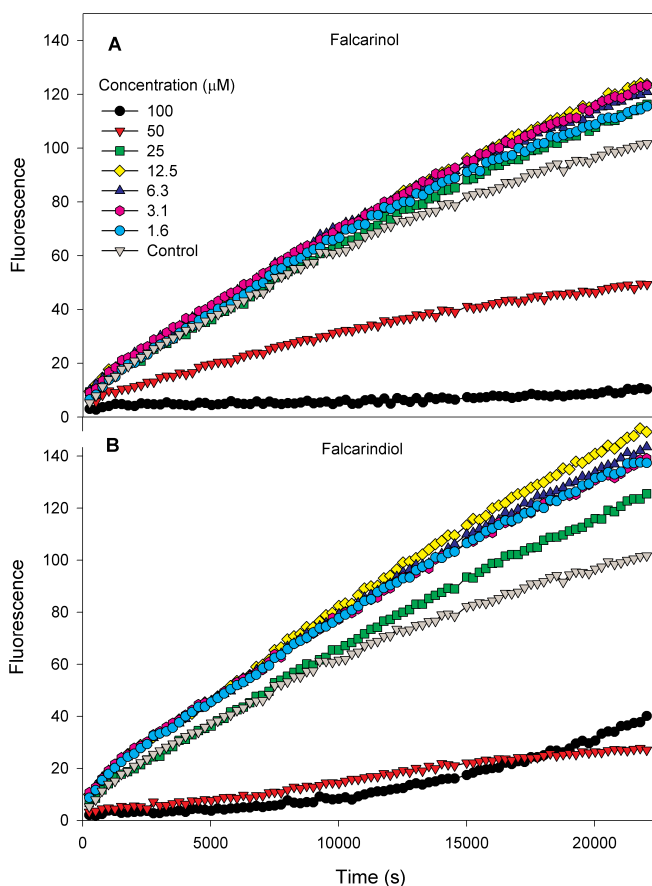


**FIGURE 2.** LSMs ( $n = 4$ ) of the relative mRNA expression in primary porcine myotubes exposed to  $100 \mu\text{M}$   $\text{H}_2\text{O}_2$  for 1 h after 24 h pre-exposure to falcarinol (A) or falcarindiol (B) at concentrations ranging from  $6.25 \mu\text{M}$  to  $50 \mu\text{M}$ . Cells were harvested for RNA extraction 18 h after stress exposure. Within each gene (HSP70: Heat shock protein 70, HO1: Hemoxygenase 1, cGPx: glutathione peroxidase), within each compound points without a common letter are significantly different ( $P < 0.05$ ).

## RESULTS

### mRNA expression

The mRNA expression of both the heat shock proteins HSP70 and HO1 of  $\text{H}_2\text{O}_2$ -stressed myotubes were lower in myotubes pre-incubated with  $6.25 \mu\text{M}$  falcarinol (Figure 2 A) or falcarindiol (Figure 2 B) compared to control cells exposed to  $\text{H}_2\text{O}_2$  without any pre-treatment of the polyacetylenes. Increasing the concentration of the polyacetylenes in the pre-incubation to  $12.5 \mu\text{M}$  further decreased the mRNA expression of both heat shock proteins whereas a further increased concentration to  $25 \mu\text{M}$  caused no (falcarinol) or only slightly (falcarindiol) further reduc-



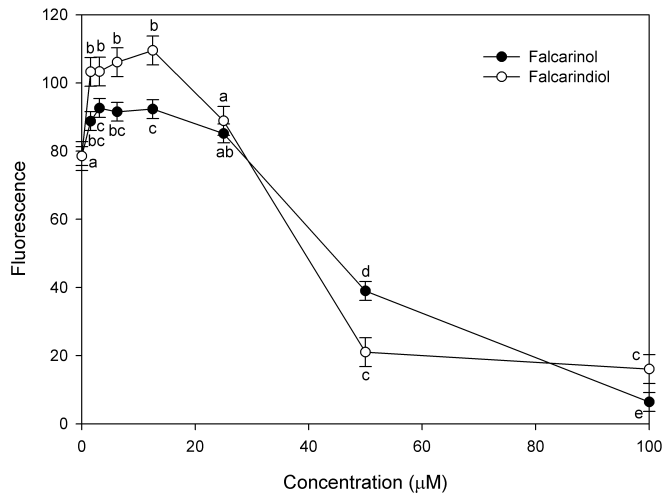
**FIGURE 3.** LSMeans ( $n = 3$ ) of oxidation of intracellular 2,7-dichlorofluorescein in primary porcine myotubes exposed to 100  $\mu\text{M}$   $\text{H}_2\text{O}_2$  after 24 h pre-exposure to falcarinol (A) or falcarindiol (B) at concentrations ranging from 1.6  $\mu\text{M}$  to 100  $\mu\text{M}$ .

tion in the mRNA expression of HSP70 and HO1. However, both heat shock proteins had increased mRNA expression when pre-incubated with 50  $\mu\text{M}$  of either polyacetylene compared to that of the expression at 25  $\mu\text{M}$  reaching at least the levels of cells pre-incubated with only 6.25  $\mu\text{M}$  polyacetylene. The mRNA expression of glutathione peroxidase (cGPx) followed the reverse pattern as it increased at polyacetylene concentrations of 25  $\mu\text{M}$  or below and declined to at least the level of cells pre-incubated with only 6.25  $\mu\text{M}$  polyacetylene when preincubated with 50  $\mu\text{M}$  polyacetylene.

### DCFH<sub>2</sub> oxidation

The intracellular oxidation of DCFH<sub>2</sub> in myotubes exposed to  $\text{H}_2\text{O}_2$  with or without pre-incubation with polyacetylenes is shown in Figure 3



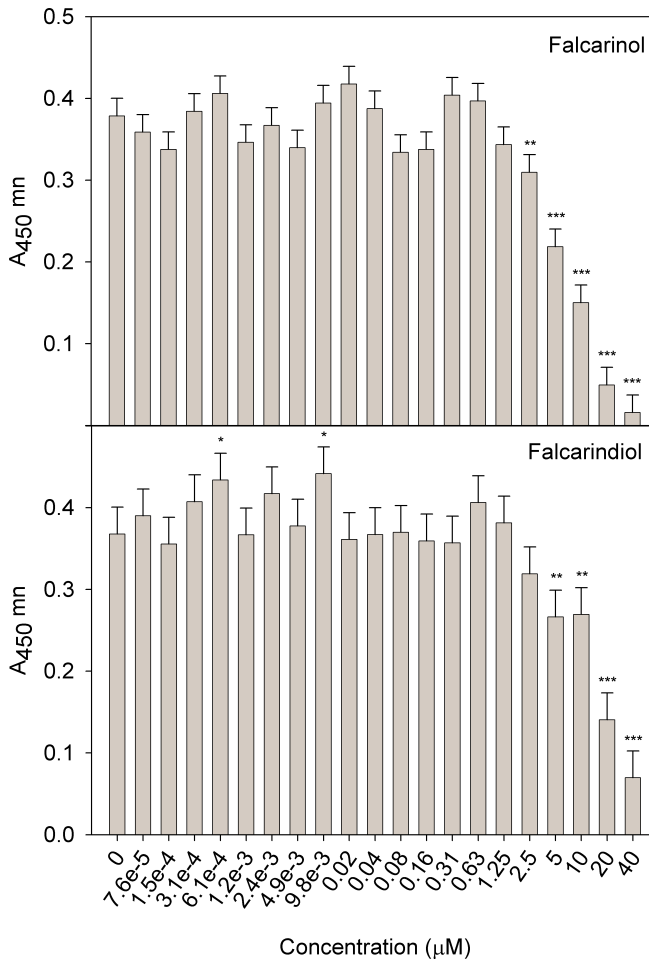


**FIGURE 4.** LSMs (n = 3) and SEM of oxidation of intracellular 2,7-dichlorofluorescein in primary porcine myotubes exposed to 100 μM H<sub>2</sub>O<sub>2</sub> for 4 h (one selected time-point from Figure 3) after 24 h pre-exposure to falcarinol (A) or falcarindiol (B) at concentrations ranging from 1.6 μM to 100 μM. Within each polyacetylene, points without a common letter are significantly different (P < 0.05).

with continuous measurements every 4 minutes, and a selected time point at 4 h is illustrated in Figure 4. In control myotubes exposed to H<sub>2</sub>O<sub>2</sub> without pre-incubation with polyacetylenes the DCFH<sub>2</sub> oxidation increased over the assay period, as illustrated in Figure 3. Myotubes pre-incubated with either falcarinol or falcarindiol in the concentration range from 1.6-12.5 μM accelerated the DCFH<sub>2</sub> oxidation (Figure 3) resulting in an increased value at all time points (Figure 4). However, upon pre-incubation with 50 or 100 μM of either polyacetylene the DCFH<sub>2</sub> oxidation was decreased compared to that of the control myotubes exposed to H<sub>2</sub>O<sub>2</sub> without any polyacetylene pre-incubation

### Myoblast viability

Viability of myoblasts in the presence of various concentrations of the polyacetylenes falcarinol and falcarindiol are shown in Figure 5 A and B, respectively. Compared to the control myoblasts without polyacetylene addition the viability of myoblasts was significantly reduced in the presence of falcarinol and falcarindiol in concentrations above 2.5 and 5 μM, respectively. At lower concentrations (9.8 and 0.61 nM) of falcarindiol the myoblast viability was significantly increased, and even though the concentrations in between were not significantly increased this may indicate a similar trend as previously indicated in studies on other cell types (Hansen et al. 2003; Young et al. 2007) where proliferation was increased at low concentrations of falcarinol.



**FIGURE 5.** LSMeans (n = 4) and SEM of A<sub>450 nm</sub> (WST-1 assay) expressing viable myoblasts upon exposure to falcarinol (A) or falcarindiol (B) for 48 h at concentrations ranging from 7.6 10<sup>-5</sup> µM to 40 µM. Values significantly different from control are indicated by \* P < 0.05 or \*\*\* P < 0.001.

## DISCUSSION

In order to allow the investigation of biphasic responses of isolated plant compounds on cellular stress responses in myotube cultures we exposed myotubes to a mild and reversible stress condition. The myotubes showed an increased intracellular ROS production (DCFH<sub>2</sub> oxidation) when exposed to 100 µM H<sub>2</sub>O<sub>2</sub>, and this cellular ROS formation may be comparable to that of contracting muscle cells under moderate physical exercise which acts as health beneficial signals to increase the defense systems i.e. up-regulation of the anti-oxidative enzymes (Gomez-Cabrera et al. 2008). Gomez-Cabrera et al. also argued that only at the point where the amount of ROS generation exceeds the capacity of

the cellular defense mechanisms the cells are adversely affected by the oxidative stress. Hence, the extent of intracellular ROS generation would determine if the ROS has an overall beneficial or harmful effect on the cells. The isolated plant compounds falcarinol and falcarindiol have proven to be very bioactive (Hansen et al. 1986; Bernart et al. 1996; Zidorn et al. 2005; Christensen and Brandt 2006); and in addition these compounds may interfere with the intracellular ROS production, quenching of these or ROS signalling functions. In the present study pre-exposure to low concentrations ( $< 25 \mu\text{M}$ ) of falcarinol and falcarindiol accelerated the intracellular oxidation of DCFH<sub>2</sub>, indicating an increased formation of reactive oxygen species (ROS) within the myotubes (Figures 3 and 4) and this co-occurred with increased m-RNA expression of the anti-oxidative enzyme glutathione peroxidase (cGPx), which in this context may be regarded as an indicator of an adaptive action towards the increased intracellular ROS content. This adaptive action also co-occurred with a decreased mRNA expression of the heat shock proteins HSP70 and HO1. The HSP70 is cytoprotective and acts as a chaperone protein assisting the folding of newly produced proteins as well as solubilising denatured protein aggregates, restore protein function and assist clearance of damaged proteins (Kiang and Tsokos 1998), whereas HO1 is a heme degrading enzyme that binds to metalloporphyrins (Maines 1988). Thus, the need for protective and repairing actions of the HSPs seemed to be reduced at low concentrations of polyacetylenes compared to control cells exposed to H<sub>2</sub>O<sub>2</sub> without pre-exposure to polyacetylenes. The decreased need for cellular HSPs may be a reflection of a reduced demand for protection and repair within the myotubes as a consequence of less damaging actions due to the adaptation (e.g. increased cGPx) to the moderately increased ROS. Hence, polyacetylenes in the low concentration range seem to have a protective role as they trigger parts of the defense mechanisms through a slightly increased ROS generation. Other studies have also shown suppression of the HSP60 and HSP70 response in lymphocytes challenged with H<sub>2</sub>O<sub>2</sub> (Khassaf et al. 2003). DCFH<sub>2</sub> oxidation decreases below that in the control myotubes when exposed to polyacetylene concentrations above 25  $\mu\text{M}$ , accompanied by decreased glutathione peroxidase mRNA expression and an increased mRNA expression of the HSPs compared to that in myotubes pre-exposed to polyacetylenes at lower concentrations. The mechanism by which high concentrations of polyacetylenes affect the myotubes may be either by causing a reduction in the generation of ROS or by quenching the already produced ROS, leading to a hampered initiation of parts of the defense mechanisms within the myotubes as indicated by the decreased cGPx mRNA expression, and a simultaneously increased demand for the protective/repairing HSPs as the mRNA expressions for these proteins increase. This effect may be comparable to the negative effects of antiox-

idants in the context of moderate exercise, where these compounds hamper adaptive actions towards ROS as recently discussed by Jackson (2008) and Gomez-Cabrera et al. (2008). Alternatively, the high concentrations of faltarinol and faltarindiol used in the present study may have had a distinct toxic effect on the myotubes, by which several cellular responses and pathways are being compromised. These biphasic effects may be encompassed by the hormesis concept, which is defined by a U-shaped dose-response relationship (Calabrese 2003), e.g. a low dose stimulatory effect combined with a high dose inhibitory effect (Calabrese and Baldwin 2003). This phenomenon was also proven in the CaCo-2 cells exposed to faltarinol where DNA strand breakage and expression of the apoptosis indicator caspase-3 active protein was reduced at low concentrations of faltarinol and increased at high concentrations (Young et al. 2007). The bell-shaped dose-response found in proliferation assays of bovine mammary epithelial cells (Hansen et al. 2003) and CaCo-2 cells (Young et al. 2007) exposed to faltarinol, supports the indication of increased viability of myoblasts in a narrow low concentration-range and a decreased viability at high doses. In conclusion, pre-incubation of myotubes with low concentrations of the polyacetylenes faltarinol and faltarindiol prior to H<sub>2</sub>O<sub>2</sub> exposure induced a protective effect as it triggered parts of the defense mechanisms whereas higher concentrations of the polyacetylenes had adverse effects.

## ACKNOWLEDGMENTS

We thank The Danish Research Agency, Ministry of Science Technology and Innovation for financial support.

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