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RESEARCH NOTE

REVISED Strigolactone GR24 upregulates target genes of the cytoprotective transcription factor Nrf2 in skeletal muscle [version 2; referees: 2 approved]

Previously titled: Strigolactone GR24 upregulates Nrf2 target genes and may protect against oxidative stress in skeletal muscle

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Abstract

GR24 is a synthetic strigolactone analog, demonstrated to regulate the development of plants and arbuscular mycorrhizal fungi. GR24 possesses anti-cancer and anti-apoptotic properties, enhances insulin sensitivity and mitochondrial biogenesis in skeletal myotubes, inhibits adipogenesis, decreases inflammation in adipocytes and macrophages and downregulates the expression of hepatic gluconeogenic enzymes. Transcription factor Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) is a master regulator of antioxidant response, regulating a multitude of genes involved in cellular stress responses and anti-inflammatory pathways, thus maintaining cellular redox homeostasis. Nrf2 activation reduces the deleterious effects of mitochondrial toxins and has multiple roles in promoting mitochondrial function and dynamics. We studied the role of GR24 on gene expression in rat L6 skeletal muscle cells which were differentiated into myotubes. The myotubes were treated with GR24 and analyzed by microarray gene expression profiling. GR24 upregulated the cytoprotective transcription factor Nrf2 and its target genes, activating antioxidant defences, suggesting that GR24 may protect skeletal muscle from the toxic effects of oxidative stress.

Keywords

Strigolactone, GR24, Nrf2, Oxidative stress, Microarray

Open Peer Review

Referee Status: ✔ ✔

	Invited Referees	
	1	**2**
REVISED **version 2** published 14 Nov 2018	✔ report	✔ report
version 1 published 13 Sep 2018	? report	? report

1 **David A. Hood**, York University, Canada

2 **Tugba Boyunegmez Tumer**, Çanakkale Onsekiz Mart University, Turkey

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Comments (0)

Corresponding author: Shalem Raju Modi (shalem.modi@uef.fi)

Author roles: Modi SR: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; **Kokkola T**: Conceptualization, Data Curation, Formal Analysis, Investigation, Methodology, Project Administration, Resources, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Introduction

Strigolactones are carotenoid-derived phytohormones with endogenous roles in regulating plant growth and exogenous roles in establishing symbiosis of host plant with arbuscular mycorrhizal fungi[1]. Strigolactones induce beneficial effects in mycorrhiza, such as mitochondrial biogenesis and ATP production[2–4]. The anti-cancer properties[5–8] and anti-inflammatory potential[9] of strigolactones have recently been investigated in mammalian cells. The positive effects of strigolactone analog GR24 in enhancing insulin sensitivity, mitochondrial function and inhibiting adipogenesis and inflammation in insulin-sensitive cells have also been demonstrated[10,11].

Nrf2 (Nuclear factor (erythroid-derived 2)-like 2) activates gene transcription by binding to the antioxidant response element (ARE) in the promoters of its target genes. It regulates multiple biological functions ranging from cellular redox metabolism, detoxification, heme, lipid and glucose metabolism, NADPH generation, autophagy, apoptosis, xenobiotic stress reponse to inflammation by interacting with its target genes, regulating an extensive antioxidant protein network. Nrf2 is associated with disease pathologies like cancer[12], hepatotoxicity[13], cardiovascular disease[14] and neurodegenerative diseases[15].

Mitochondria are the major sites of reactive oxygen species (ROS) production and also the targets of their toxic effects. Mitochondrial dysfunction has been associated with the development of insulin resistance, and diabetes is known to induce oxidative stress through the overproduction of ROS and ROS-induced DNA damage[16]. Nrf2 activation defends against mitochondrial toxins and ROS and affects mitochondrial function by regulating mitochondrial biogenesis, mitochondrial fatty acid oxidation, respiration, ATP production and mitochondrial dynamics[17]. With its versatile protective mechanism against oxidative stress, pancreatic β-cell apoptosis and insulin resistance, Nrf2 has become a promising therapeutic target for the treatment of type 2 diabetes[18].

We have demonstrated that GR24 ameliorates insulin sensitivity, stimulates mitochondrial biogenesis and ATP production and upregulates genes regulating mitochondrial function in L6 myotubes[10]. Very recently, the efficacy of GR24 in promoting cytoprotective responses via Nrf2 activation was reported in hepatic and macrophage cell lines[19]. This work reports a transcriptomic study revealing the potential beneficial effects of GR24 in upregulating Nrf2 and its target genes involved in detoxification, carbohydrate and lipid metabolism, heme metabolism, NADPH regeneration and oxidative stress in L6 myotubes, thus contributing to metabolic homeostasis.

Methods

Results from the methods described in this study have been published previously[10], although the analyses described here are published for the first time.

Cell culture and differentiation

Rat L6 myoblasts (ATCC, Manassas, VA, USA) were maintained in Dulbecco's modified Eagle's medium (DMEM 4.5 g/l glucose, Lonza, Basel, Switzerland) supplemented with 10% FBS (Hyclone, Pasching, Austria), 2 mM L-glutamine (Lonza) and 1% penicillin/streptomycin (Lonza) at +37°C in a humidified atmosphere of 5% CO_2. The cells were seeded in multiwell plates at 2×10^4 cells/cm^2 one day before starting the differentiation. Myoblasts were differentiated into myotubes by switching into α-MEM media (Gibco, Paisley, UK) supplemented with 2% horse serum (Gibco) and 1% penicillin/streptomycin. GR24 (3E,3aR,8bS)-3-[[(2S)-4-methyl-5-oxo-2H-furan-2-yl] oxymethylidene]-4,8b-dihydro-3aH-indeno[1,2-b]furan-2-one, Chiralix, Nijmegen, Netherlands) was dissolved in DMSO. The control samples had equivalent DMSO concentration.

Microarray sample preparation

L6 myotubes were treated with 60 µM GR24 at 7 days of differentiation for 24 h in three independent experiments, resulting in three replicate microarrays in each treatment group. Total RNAs were extracted with RNeasy Mini kit (Qiagen, Hilden, Germany) and RNA quality was assessed with the Agilent Bioanalyzer 2100 (Agilent Technologies, Espoo, Finland). Total RNA (200 ng) was converted to cDNA with Agilent AffinityScript RNase Block, labelled according to manufacturer's instructions and purified using RNeasy mini spin columns (Qiagen). RNA Spike-In Kit (Exiqon, Vedbaek, Denmark) was used to monitor the success of labelling. Samples were then mixed with blocking agent, fragmentation and hybridization buffer, and were hybridized to Agilent SurePrint G3 Rat GE 8×60 K Microarrays for 17 h at 65°C before washing and scanning with Agilent Scanner G2505C using manufacturer's protocols. Agilent Feature Extraction software was used to extract data from raw microarray image files[10].

Microarray data processing

The data was processed with limma package (version 3.28.21) of the Bioconductor software[20]. Microarray data are MIAME compliant. Differentially expressed transcripts were analysed by Bayes moderated t-statistics followed by the Benjamini-Hochberg correction method to control false discovery rate (FDR) with significance threshold set at $p < 0.05$[20,21].

Results and discussion

The top 5 GR24-upregulated genes (glutathione S-transferase alpha 1, metallothionein 1M, heme oxygenase 1, glutathione S-transferase alpha 2, and sequestosome 1) were found to be known targets of Nrf2 (Table 1). As the prototypical Nrf2 target gene NAD(P)H quinone dehydrogenase 1 (Nqo1) was also found high on the list, it was compelling to look into the extensive list of Nrf2 target genes. We found 56 known Nrf2 target genes[22], including Nrf2 itself, to be upregulated by GR24 treatment

Table 1. Selected upregulated Nrf2 target genes in L6 myotubes after 24 h treatment with 60 µM GR24.

Gene symbol	Description	Accession	logFC	Fold change	Adjusted P value
Detoxication: Phase I drug oxidation, reduction and hydrolysis					
Adh7	Alcohol dehydrogenase 7	NM_134329	1.604	3.04	1.746E-09
Akr1b1	Aldo-keto reductase family 1 member B1	NM_012498	1.831	3.56	7.986E-09
Akr1b8	Aldo-keto reductase family 1. member B8	NM_173136	2.503	5.67	1.254E-10
Akr1cl	Aldo-keto reductase family 1. member C-like	NM_001109900	0.685	1.61	7.675E-06
Cbr1	Carbonyl reductase 1	NM_019170	1.060	2.08	3.055E-08
Nqo1	NAD(P)H quinone dehydrogenase 1	NM_017000	3.144	8.84	1.602E-10
Ptgr1	Prostaglandin reductase 1	NM_138863	3.233	9.40	1.196E-09
Detoxication: Phase II drug conjugation					
Gsta1	Glutathione S-transferase alpha 1	NM_031509	4.610	24.42	3.458E-11
Gsta2	Glutathione S-transferase alpha 2	NM_001010921	3.676	12.78	1.348E-09
Gstm1	Glutathione S-transferase mu 1	NM_017014	0.707	1.63	7.461E-06
Gstp1	Glutathione S-transferase pi 1	NM_012577	2.498	5.65	3.862E-10
Mgst1	Microsomal glutathione S-transferase 1	NM_134349	2.327	5.02	1.638E-09
Ugt1a2	UDP glucuronosyltransferase 1 family. polypeptide A2	NM_001039691	0.169	1.12	3.123E-02
Detoxication: Phase III drug transport					
Abcb6	ATP binding cassette subfamily B member 6	NM_080582	0.194	1.14	6.514E-03
Abcc1	ATP binding cassette subfamily C member 1	NM_022281	1.420	2.68	2.985E-07
Abcc2	ATP binding cassette subfamily C member 2	NM_012833	0.653	1.57	2.263E-03
Abcc4	ATP binding cassette subfamily C member 4	NM_133411	1.367	2.58	4.359E-07
Abcc5	ATP binding cassette subfamily C member 5	NM_053924	0.807	1.75	4.986E-06
Antioxidant systems					
Cat	Catalase	NM_012520	2.205	4.61	7.440E-09
Gclc	Glutamate-cysteine ligase, catalytic subunit	NM_012815	1.231	2.35	2.616E-07
Ggt1	Gamma-glutamyltransferase 1	NM_053840	2.590	6.02	6.774E-09
Glrx	Glutaredoxin	NM_022278	0.717	1.64	9.108E-05
Gls	Glutaminase	NM_012569	1.782	3.44	2.165E-09
Gpx4	Glutathione peroxidase 4	NM_001039849	0.451	1.37	1.766E-05
Prdx1	Peroxiredoxin 1	NM_057114	1.361	2.57	1.274E-06
Prdx6	Peroxiredoxin 6	NM_053576	1.291	2.45	9.933E-06
Slc6a9	Solute carrier family 6 member 9 (glycine transporter)	NM_053818	2.283	4.87	9.548E-10
Slc7a11	Solute carrier family 7 member 11 (cystine/glutamate transporter)	NM_001107673	0.870	1.83	5.449E-06
Sod1	Superoxide dismutase 1	NM_017050	0.939	1.92	2.031E-06
Srxn1	Sulfiredoxin 1	NM_001047858	2.330	5.03	7.817E-10
Txn1	Thioredoxin	NM_053800	1.712	3.28	4.093E-09
Txnrd1	Thioredoxin reductase 1	NM_031614	2.193	4.57	1.765E-08

Gene symbol	Description	Accession	logFC	Fold change	Adjusted P value
Carbohydrate metabolism and NADPH regeneration					
G6pd	Glucose-6-phosphate dehydrogenase	NM_017006	2.184	4.54	7.894E-09
Idh1	Isocitrate dehydrogenase 1	NM_031510	1.096	2.14	4.304E-08
Me1	Malic enzyme 1	NM_012600	0.860	1.81	8.162E-08
Pgd	Phosphogluconate dehydrogenase	NM_001305435	1.920	3.79	1.503E-09
Taldo1	Transaldolase 1	NM_031811	1.150	2.22	2.143E-07
Tkt	Transketolase	NM_022592	0.989	1.99	2.321E-06
Ugdh	UDP-glucose 6-dehydrogenase	NM_031325	0.623	1.54	2.451E-05
Lipid metabolism					
Acot7	Acyl-coa thioesterase 7	NM_001146061	0.852	1.80	6.273E-07
Acox1	Acyl-coa oxidase 1	NM_017340	0.706	1.63	1.897E-05
Ces1a	Carboxylesterase 1A	NM_001190375	1.132	2.19	7.021E-05
Pnpla2	Patatin-like phospholipase domain-containing 2	NM_001108509	0.424	1.34	7.480E-05
Heme and iron metabolism					
Blvra	Biliverdin reductase A	NM_053850	0.890	1.85	1.973E-06
Blvrb	Biliverdin reductase B	NM_001106236	1.497	2.82	5.723E-09
Fech	Ferrochelatase	NM_001108434	1.431	2.70	5.570E-09
Fth1	Ferritin heavy chain 1	NM_012848	0.877	1.84	4.826E-07
Ftl1	Ferritin light chain 1	NM_022500	1.017	2.02	3.213E-07
Hmox1	Heme oxygenase 1	NM_012580	3.959	15.55	1.136E-10
Transcription factors and associated proteins					
Ahr	Aryl hydrocarbon receptor	NM_013149	0.867	1.82	1.519E-06
Cebpb	CCAAT/enhancer binding protein beta	NM_001301715	0.959	1.94	1.102E-06
Mafg	Mafg protein	NM_022386	0.718	1.64	2.006E-05
Mt1m	Metallothionein 1M	NM_001137564	4.009	16.10	7.970E-10
Nfe2l2	Nuclear factor, erythroid 2-like 2 (Nrf2)	NM_031789	0.659	1.58	3.116E-05
Rxra	Retinoid X receptor alpha	NM_012805	0.788	1.73	1.677E-05
Sqstm1	Sequestosome 1	NM_181550	3.576	11.93	3.458E-11
Ubiquitin ligase substrate adaptor					
Keap1	Kelch-like ECH-associated protein 1	NM_057152	1.269	2.41	3.765E-08

(Table 1). Prior to our study, the effects of strigolactones treatment on the mammalian cell transcriptome have only been investigated in human osteosarcoma cells, where strigolactone analogs mainly upregulated the heat shock stress proteins and downregulated the cell cycle[5].

Activation of Nrf2 signaling is known to have beneficial effects in cancer, metabolic syndrome, obesity, nephropathy, retinopathy, neuropathy, and β-cell protection. Nrf2 regulates the transcription of genes involved in antioxidant, detoxification and metabolic processes[23]. In our study, GR24 enhanced the expression of the Nrf2-dependent antioxidant genes Nqo1 and heme oxygenase 1, which are known to block inflammatory pathways[24]. GR24 has recently been shown to alleviate inflammation and upregulate these two NRF2 target genes in hepatic and macrophage cells[19].

Glutathione and associated enzymes form an important antioxidant defence system. Treatment with GR24 was found to increase root glutathione content in plants[25], but there are no previous

reports on the effects of strigolactones on the glutathione system in mammalian cells. Our results show that GR24 treatment upregulated glutamate-cysteine ligase, the rate-limiting enzyme in glutathione synthesis (Table 1). Many components of the glutathione system are upregulated by Nrf2, and the elevations in the expression of glutaredoxin, glutathione peroxidase 4 and several glutathione S-transferases in GR24-treated cells were evident in our results (Table 1).

Conclusions

GR24 upregulated the cytoprotective transcription factor Nrf2 and its target genes, which are involved in detoxification, antioxidant systems, carbohydrate and lipid metabolism, heme and iron metabolism, NADPH regeneration and regulation of transcription in L6 myotubes. Future research aimed to elucidate the effects of GR24 in the oxidative stress mechanisms in skeletal muscle at the protein level may provide supporting evidence about the therapeutic potential of this compound.

Data availability

Microarray raw data have been deposited in the NCBI Gene Expression Omnibus (GEO), accession number GSE90833: https://identifiers.org/geo/GSE90833.

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Open Peer Review

Current Referee Status: ✔ ✔

Version 2

Referee Report 24 January 2019

https://doi.org/10.5256/f1000research.18592.r40629

✔ **David A. Hood**
Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, Canada

I have reviewed the responses and am satisfied with the responses. I have no further comments to add.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Referee Report 14 November 2018

https://doi.org/10.5256/f1000research.18592.r40630

✔ **Tugba Boyunegmez Tumer**
Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

Authors have carried out satisfactory changes in the revised MS. In this form, it can be acceptable for indexing.

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Referee Report 31 October 2018

https://doi.org/10.5256/f1000research.17660.r39708

❓ **Tugba Boyunegmez Tumer**
Department of Molecular Biology and Genetics, Çanakkale Onsekiz Mart University, Çanakkale, Turkey

In current research note, a microarray study was carried out to analyze the effect of GR24, a synthetic strigolactone analog, on the gene expression profile of rat L6 myotubes. 60 µM of GR24 was used to treat L6 myotubes at 7th day of differentiation for 24 h in three independent experiments. Microarray data was processed and differentially expressed transcripts were analyzed. Upregulated genes were partly presented in Table 1. Accordingly, GR24 upregulated the cytoprotective transcription factor Nrf2 and its several target genes involved in antioxidant defense and phase II detoxification processes. The fold values especially for *GSTa1* and *a2*, *NQO1*, *GGt1*, *Hmox1*, *Mtm1m*, *Sqstm1* were considerably and significantly high (above 6 fold). Authors previously showed that GR24 (60 µM) ameliorates insulin sensitivity, stimulates mitochondrial biogenesis and ATP production and upregulates genes regulating mitochondrial function in L6 myotubes (Modi et al., 2017[1]).

Also, in this previous report it was shown that 9838 transcripts were upregulated and 6315 transcripts were downregulated in L6 myotubes as a result of 60 µM GR24 treatment. Up and down regulated transcripts involved in insulin signaling and mitochondrial function in L6 myaotubes have been presented in this previous paper (Modi et al., 2017[1]). In the present research note, authors represented up regulated transcripts involved in oxidative stress and cytoprotective signaling when myotubes were treated with 60 µM GR24. Since the work can be classified as original and the results are remarkable, data deserves to be published in F1000Research as research note. However, the data is limited to reach the conclusion suggesting that GR24 may protect skeletal muscle from the toxic effects of oxidative stress and thus contributing to metabolic homeostasis. In the scope of report, there is no any biological assay or disease modelling evaluating the beneficial effects of GR24 on myotubes. Furthermore, gene expression profiling does not always reflect the changes on the protein level. Regarding these facts, title also should not contain the assumption suggesting that GR24 may protect against oxidative stress in skeletal muscle.

In my opinion, both conclusion and title need revision and if possible it is better to represent additional data and interpretation in terms of down regulated genes in specified signaling pathways. Also could you please add an explanation about why you chose 60 µM concentration?

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PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

> Author Response 07 Nov 2018
>
> **SHALEM RAJU MODI**, University of Eastern Finland, Finland
>
> **We thank the referee for kindly evaluating our research note and for the valid comments and suggestions. We agree with the comments and hence, we changed the discussion part and also the title of the manuscript.**
>
> Also could you please add an explanation about why you chose 60 µM concentration?
>
> **We initially tested GR24 at various concentrations from 20 µM – 100 µM. We observed that at 60 µM concentration, GR24 significantly upregulated SIRT1 protein expression and enhanced glucose uptake in skeletal muscle cells (Sci Rep. 2017; 7(1): 17606.). Moreover, longer exposure to 100 µM GR24 had a small but significant cytotoxic effect. Hence, we have chosen 60 µM to be an ideal concentration for all our experiments.**
>
> *Competing Interests:* No competing interests were disclosed.

Referee Report 02 October 2018

? **David A. Hood**

Muscle Health Research Centre, School of Kinesiology and Health Science, York University, Toronto, ON, Canada

Interesting investigation of the effect of a plant-based compound to change gene expression in muscle cells, however the study is very limited in scope.

1. All reported data show increases in mRNA. Are there no significant decreases that are noteworthy?
2. Have any changes in mRNA using microarrays been verified with Q-PCR? This is standard practice;
3. Could a few protein blots be included to strengthen the data and take the interpretation to the protein level?
4. The title assumes that the mRNA changes lead to protein changes which would impact oxidative stress. The title needs to be re-worded unless actual measures of oxidative stress are performed. this would be relatively easy with an oxi-blot, or ROS measure in cells.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?

Yes

If applicable, is the statistical analysis and its interpretation appropriate?

Yes

Are all the source data underlying the results available to ensure full reproducibility?

Yes

Are the conclusions drawn adequately supported by the results?

Partly

Competing Interests: No competing interests were disclosed.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 19 Oct 2018

SHALEM RAJU MODI, University of Eastern Finland, Finland

We thank the referee for carefully evaluating our research note. He has correctly identified that the study has its limitations. As we have been engaged in other studies and have not been able to dedicate more time for continuing on this track, we decided to submit our microarray analyses in the current format. This Research Note format facilitates publishing small studies, such as our study with only a single table.

1. All reported data show increases in mRNA. Are there no significant decreases that are noteworthy?

In the current short publication, we do not report all significant findings. A total of 16,153 transcripts were differentially regulated (9,838 up- and 6,315 downregulated) to the GR24 treatment. Some of the differentially regulated transcripts have been described in our previous publication (Modi S, Yaluri N, Kokkola T, et al.: Plant-derived compounds strigolactone GR24 and pinosylvin activate SIRT1 and enhance glucose uptake in rat skeletal muscle cells. *Sci Rep.* 2017; 7(1): 17606.). Nevertheless, the most interesting finding from the microarray experiments was the strong upregulation of Nrf2 target transcripts by GR24 treatment.

2. Have any changes in mRNA using microarrays been verified with Q-PCR? This is standard practice;

3. Could a few protein blots be included to strengthen the data and take the interpretation to the protein level?

4. The title assumes that the mRNA changes lead to protein changes which would impact oxidative stress.The title needs to be re-worded unless actual measures of oxidative stress are performed. this would be relatively easy with an oxi-blot, or ROS measure in cells.

These are very valid questions. The suggested qPCR analyses, protein blots and measures of oxidative stress would be essential next steps to verify the changes in gene expression. For that reason, we published our preliminary analyses as a Research Note, as additional analyses would have been required in order to produce a complete full-length article. When drafting the title, we avoided making strong claims on the effects on oxidative stress. After reading the suggestions by the referee, we have reconsidered the title, and will submit a new version of the article with a title "Strigolactone GR24 upregulates target genes of the cytoprotective transcription factor Nrf2 in skeletal muscle".

Competing Interests: No competing interests were disclosed.

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