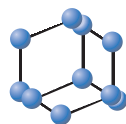


RESEARCH ARTICLE

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SCIENCE

IRS-1 pY612 and Akt-1/PKB pT308 Phosphorylation and Anti-inflammatory Effect of Diindolylmethane in Adipocytes Cocultured with Macrophages



Alfonso López-Vázquez¹, Jesús J. Garcia-Bañuelos^{2,*}, Angélica S. González-Garibay¹, Pedro E. Urzúa-Lozano¹, Susana Del Toro-Arreola¹, Miriam R. Bueno-Topete¹, Sergio Sánchez-Enríquez³, José F. Muñoz-Valle⁴, Luis F. Jave-Suárez⁵, Juan Armendáriz-Borunda² and Blanca E. Bastidas-Ramírez^{1,*}

¹Instituto de Investigación en Enfermedades Crónicas Degenerativas, Departamento de Biología Molecular y Genómica, Universidad de Guadalajara, Guadalajara, Jalisco, México; ²Instituto de Biología Molecular en Medicina y Terapia Génica, Departamento de Biología Molecular y Genómica, Universidad de Guadalajara, Guadalajara, Jalisco, México; ³Laboratorio de Bioquímica, Departamento de Biología Molecular y Genómica, Universidad de Guadalajara, Guadalajara, Jalisco, México; ⁴Instituto de Investigación en Ciencias Biomédicas, Departamento de Biología Molecular y Genómica, Universidad de Guadalajara, Guadalajara, Jalisco, México; ⁵Centro de Investigación Biomédica de Occidente, Instituto Mexicano del Seguro Social, Guadalajara, Jalisco, México

Abstract: Background: 3,3'-Diindolylmethane (DIM) is a condensation product of indole-3-carbinol, a glucosinolate naturally occurring in *Brassica* genus vegetables. The antiinflammatory properties of DIM through the inhibition of NF- κ B, as well as its ameliorating effects on glucose tolerance and hyperglycemic states, have been described. A subclinical proinflammatory profile resultant from the interaction of adipocytes and macrophages has been reported in obesity, affecting the insulin signaling pathway, contributing to insulin resistance.

Objective: The aim of this study was to evaluate the effect of DIM on proinflammatory cytokines and phosphorylation of IRS-1 pY612 and Akt-1/PKB pT308 in an obesity-induced inflammation model.

Methods: Differentiated 3T3-L1 adipocytes were co-cultured with RAW 264.7 macrophages and exposed to 20 μ M, 40 μ M and 60 μ M DIM for 24 h followed by 100 nM insulin for 20 min. MCP-1, IL-6 and TNF α were quantified in the supernatant through individual ELISAs. Adipocyte lysates were used to determine the relative expression of the proinflammatory mediators by qPCR, and the phosphorylation of IRS-1 pY612 and Akt-1/PKB pT308 proteins by western blot analysis.

Results: DIM significantly ($p < 0.05$) reduced the production and mRNA expression of MCP-1, IL-6, and TNF α in a DIM concentration dependent manner, concomitantly increasing the abundance of IRS-1 pY612 and Akt-1/PKB pT308.

Conclusion: Our results suggest that DIM influences the insulin transduction pathway by exerting an antiinflammatory effect. The potential therapeutic benefits of DIM in the treatment of glucose metabolic disorders deserve further studies.

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*Address correspondence to these authors at the Instituto de Investigación en Enfermedades Crónicas Degenerativas, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, puerta 7, 2do. piso., Calle Sierra Mojada # 950, Col. Independencia, Guadalajara, Jalisco, C.P. 44340, México; Tel: (+52) (33)1058 5296; E-mail: bastidas@cencar.udg.mx; Instituto de Biología Molecular en Medicina y Terapia Génica, Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara, puerta 7, 3er. piso, Calle Sierra Mojada # 950, Col. Independencia, Guadalajara, Jalisco, C.P. 44340, México; Tel: (+52) (33)1058 5284; E-mail: bgarcia@cucs.udg.mx

1. INTRODUCTION

Indole-3-carbinol (I3C) (C_9H_9NO), is an autolysis product of glucosinolates naturally occurring in cruciferous vegetables of the genus *Brassica*, which includes broccoli, cauliflower, Brussels sprouts, kale, turnips, collard greens, kohlrabi, cabbage and mustard rutabaga [1, 2]. 3,3'-Diindolylmethane (DIM) results from the condensation of I3C when exposed to acidic pH, and has been identified as the responsible molecule of the biological effects exhibited by I3C [3-9]. DIM

properties against diverse types of cancer, have been widely evidenced [6, 10-15]. It is believed that this biological effect is exerted by suppressing the transcriptional activity of nuclear factor- κ B (NF- κ B) [9]. Also, decreased production of TNF α and interleukin 6 (IL-6) has been observed in cultures of adipocytes [16], macrophages [17], and in co-cultures of adipocytes and macrophages treated with DIM [18]. Recently, the ameliorating effect of DIM on obesity, glucose tolerance, TNF α , IL-6, insulin, leptin, or adiponectin levels, and hyperglycemic states, has been described [7, 16, 18, 19]. On the other hand, a subclinical proinflammatory profile characterized by high levels of TNF α , IL-6 and MCP-1, has been identified in obesity [20, 21]. There is evidence in obese humans [22] and animals [23] that the interaction of adipocytes with macrophages increases the levels of proinflammatory cytokines, altering the concentration and phosphorylation of IRS-1 and protein kinase B (Akt-1/ PKB), leading in turn to insulin resistance (IR) [23-25]. Modulating inflammatory responses in obese patients may be a useful approach for preventing or ameliorating obesity-related morbidities [26]. DIM activity on glucose metabolism and inflammatory processes has been studied; however, the effects of DIM on molecules of the insulin signaling pathway associated to the inflammatory state have not been investigated. The aim of this study was to evaluate the effect of DIM on proinflammatory mediators and the concentration of IRS-1 pY612 and Akt-1/PKB pT308 in an *in vitro* model of obesity-induced inflammation and IR, consisting in the indirect co-culture of adipocytes and macrophages [27].

2. MATERIALS AND METHODS

2.1. Reagents

DIM, dimethyl-sulfoxide (DMSO), insulin, dexamethasone, isobutyl-methylxanthine (IBMX) and RIPA buffer were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium-high glucose (DMEM-HG), phosphate buffered saline solution (PBS), fetal bovine serum (FBS), non-essential amino acids and antibiotic-antimycotic solutions from Gibco Life-technologies (Grand Island, NY, USA). Enzyme linked immunosorbent assay (ELISA) kits to measure MCP-1, IL-6, and TNF- α were obtained from R&D Systems (Minneapolis MN). Trizol reagent, Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, and random primers from Invitrogen (Grand Island, NY, USA). Taqman gene expression assays, and Taqman gene expression master mix were purchased from Life Technologies Applied Biosystems (Grand Island, NY, USA). Protease, and phosphatase inhibitors (Complete minitables) were purchased from Roche Applied Science (Basel, Switzerland). Acrylamide solution, and polyvinylidene difluoride (PVDF) membranes from Bio-Rad (CA, USA). Primary antibodies anti-IRS-1 pY612 were obtained from Abcam Incorporation (Cambridge, UK). Primary antibodies: anti-Akt-1/PKB pT308, and anti-GAPDH; and secondary antibodies: HRP-conjugated anti-IRS-1 pY612 anti-mouse, anti-Akt-1/PKB pT308 anti-rabbit, and anti-GAPDH anti-mouse, from Santa Cruz Biotechnology (Dallas, Texas, USA).

2.2. Cell Culture and Differentiation of 3T3-L1 Cells

3T3-L1 and RAW264.7 cells were kindly provided by the German Cancer Research Center (Heidelberg, Germany). Cells were propagated separately in basic medium containing DMEM-HG without sodium pyruvate, supplemented with 10% FBS and 1% antibiotic-antimycotic solution (Gibco Life-Technologies). Preadipocytes were seeded in the bottom of a 6 well-plaque transwell system with a 0.4 μ m porous membrane (Corning Inc., Acton, MA) at a density of 5×10^4 cells. After two days of confluence, 3T3-L1 cells were differentiated by adding 1 μ M dexamethasone, 0.5 mM IBMX and 10 μ g/mL insulin (Sigma-Aldrich) to basic medium during 2 days, as reported before [28]. Differentiated cells were maintained during 8 days in post-differentiation medium constituted by basic medium added with 10 μ g/mL insulin (Sigma-Aldrich), replacing the medium every two days.

2.3. Coculture of 3T3-L1 Adipocytes and RAW264.7 Macrophages

Mouse 3T3-L1 pre-adipocytes were co-cultured with the mouse macrophage cell line RAW264.7 as described elsewhere [29]. Briefly, macrophages were seeded on the transwell insert over the previously differentiated 3T3-L1 cells (1×10^5 cells) on day 9 of post-differentiation at a density of 5×10^4 cells, maintaining the cultures at 37 °C in 5% CO₂, changing the post-differentiation medium every 24 h. After co-culturing for 72 h, medium was replaced by post-differentiation medium without insulin, containing different DIM concentrations for 24 h. At the end of this period, insulin was added at a concentration of 100 nM for 20 min.

2.4. DIM Assay

DIM (Sigma-Aldrich) was solubilized in DMSO (Sigma-Aldrich), and added to the co-culture chambers at a final concentration of 0 μ M DIM (control), 20 μ M, 40 μ M and 60 μ M during 24 h, maintaining the proportion of DMSO in culture medium always under 0.1%. DIM concentrations for this study were calculated based on previous reports that evidenced suppression of the inflammatory response [9, 18]. Each experiment was carried out in triplicate.

2.5. Determination of Secreted Proinflammatory Mediators

Differentiated 3T3-L1 mouse adipocytes were indirectly co-cultured with RAW-264.7 macrophages during 72 h using a transwell system, treated with 0 μ M DIM, 20 μ M, 40 μ M or 60 μ M for 24 h, and then insulin for 20 min, as described above. Cell culture supernatants were collected by centrifugation during 5 min at 10,000 x g to quantify proinflammatory molecules using individual ELISA kits (R&D systems) for MCP-1, IL-6 and TNF- α , following the manufacturer's protocols.

2.6. Reverse Transcription and Expression Assay

Total RNA was isolated from co-cultured 3T3-L1 adipocytes by removing and discarding the upper trans-well compartment containing RAW264.7 macrophages and collecting adipocytes seeded in the lower compartment of the chamber

in an eppendorf tube. Cells were lysed with trizol reagent (Invitrogen Life Technologies), according to manufacturer's protocol [30]. This modality of indirect co-culture allowed us to recover isolated adipocytes. cDNA synthesis was carried out in a volume of 20 μ L using 2 μ g total RNA, M-MLV reverse transcriptase and 0.5 mM random primers (Invitrogen Life Technologies). Subsequently, cDNA was amplified by quantitative real time polymerase chain reaction (qPCR), using Taqman probe-based gene expression assays (Applied Biosystems) as follows: (MCP-1) Mm00441242_m1; (IL-6) Mm00446190_m1; (TNF- α) Mm00443260_g1; (actin- β) Mm00607939_s1. Amplification was performed in a total reaction volume of 10 μ L containing 100 ng cDNA, 3.5 μ L H₂O, 0.5 μ L of the Taqman probe-based gene expression assay and 1X Taqman gene expression Master Mix (Applied Biosystems) in a Rotor Gene fluorescence thermal cycler (Qiagen, Limburg, NL) for 45 cycles. Each cycle consisted of denaturation at 94°C for 15 sec and annealing/extension at 60°C for 40 sec, after an initial step of 50 °C 2 min and 94 °C 10 min. Results were normalized based on the expression of actin- β gene and analyzed by the comparative relative expression CT ($2^{-\Delta\Delta C_T}$) method [31].

2.7. Determination of Intracellular Phosphorylated Proteins

After insulin exposure of co-cultures treated with different concentrations of DIM, the upper chamber with RAW264.7 cells was removed and discarded to prepare adipocyte lysates by washing twice with ice-cold PBS and adding lysis RIPA buffer (Sigma-Aldrich) containing protease and phosphatase inhibitors (Complete minitabets, Roche Applied Science). Adipocytes were lysed for 30 min at 4°C and clarified by centrifugation. Proteins were quantified by the Bradford's method and subjected to Western blot analysis by mixing 100 mg protein with loading buffer under reducing conditions. Samples were electrophoresed on 8% SDS-polyacrylamide gel and transferred to a PVDF membrane (Bio-Rad). Previously to overnight hybridization with anti-IRS-1 pY612 (1:500) (Abcam Incorporation), anti-Akt-1/PKB pT308 (1:5000) and anti-GAPDH (1:5000) (Santa Cruz Biotechnology) primary antibodies, membranes were blocked with 3% milk. Hybridization with HRP-conjugated anti-IRS-1 pY612 anti-mouse (1:500), anti-Akt-1/PKB pT308 anti-rabbit and anti-GAPDH (1:5000) anti-mouse (Santa Cruz Biotechnology) secondary antibodies, was carried out to reveal blots. A chemiluminiscent image system (Millipore, Billerica, MA, USA) was used to detect proteins. Densitometric analysis was performed by a Kodak MI 5.0 Image Analyzer (Kodak, Rochester, NY, USA). Detection of GAPDH was utilized as an internal control to assure equivalent protein load into each lane. Molecular weights of proteins were: IRS-1 pY612, 132 kDa; Akt-1/PKB pT308, 56 kDa; and GAPDH, 38 kD.

2.8. Statistical Analysis

Data were expressed as the mean \pm SD, as indicated. Statistical analyses were performed by the Kruskal-Wallis's test and differences between groups were determined by Mann-Whitney U test. A *p* value < 0.05 was considered statistically significant.

3. RESULTS

3.1. DIM Attenuates Proinflammatory Mediators in Adipocytes Co-cultured with Macrophages

Differentiated 3T3-L1 mouse adipocytes were indirectly co-cultured with RAW-264.7 macrophages during 72 h using a transwell system, exposed to 0 μ M DIM, 20 μ M, 40 μ M or 60 μ M for 24 h, followed by the addition of 100 nM insulin for 20 min, as described above. Cell culture supernatants were collected to analyze and quantify proinflammatory molecules using individual ELISA kits (R&D Systems) for MCP-1, IL-6 and TNF- α . As shown in Fig. (1), production of proinflammatory mediators significantly decreased as DIM concentration increased. MCP-1 production was statistically (*p*<0.001) lower than the control (26.4 \pm 1.1 ng/mL) at 20 μ M DIM (12.5 \pm 2.5 ng/mL), 40 μ M (16.0 \pm 2.4 ng/mL), and 60 μ M (4.9 \pm 0.4 ng/mL). Likewise, DIM significantly (*p*<0.001) decreased IL-6 production at 20 μ M (1484.7 \pm 3.8 pg/mL), 40 μ M (312.9 \pm 109 pg/mL) and 60 μ M (67.3 \pm 34.2 pg/mL) vs. the control (2848.3 \pm 124 pg/mL). TNF- α production significantly (*p*<0.01) decreased with 60 μ M of DIM (36.2 \pm 12.0 pg/mL) vs. the control (70.7 \pm 0.4 pg/mL). Paradoxically, a significant (*p*<0.01) increase in TNF- α was observed when co-cultures were treated with 20 μ M DIM (102 \pm 8.8 pg/mL) or 40 μ M (99.7 \pm 12.4 pg/mL). Maximal suppression effect on the production of MCP-1 was, IL-6, and TNF- α by 81.4 %, 97.6 % and 48.7 % respectively, compared with the control was evidenced after treatment with 60 μ M DIM.

The effect of DIM over the expression of MCP-1, IL-6 and TNF- α at the mRNA level utilizing the reverse transcription and qPCR, was also analyzed. Fig. (2) shows a downward change on the relative expression of MCP-1 at 20 μ M DIM (0.3 \pm 0.2 fold of induction; *p*<0.05), 40 μ M (0.5 \pm 0.2 fold of induction; *p*<0.05) and 60 μ M (0.2 \pm 0.02 fold of induction; *p*<0.001) vs. the control (1 \pm 0.2); and IL-6 at 20 μ M DIM (0.6 \pm 0.1 fold of induction; *p*<0.05), 40 μ M (0.3 \pm 0.1 fold of induction; *p*<0.05) and 60 μ M (0.1 \pm 0.02 fold of induction; *p*<0.001) vs. the control (1 \pm 0.2). A statistically significant decrease on the expression of TNF- α was appreciated after the exposure of the co-cultures to 60 μ M DIM (0.6 \pm 0.5 fold of induction; *p*<0.01) vs. the control (1 \pm 0.1). Contradictorily, an upward change on the relative expression of TNF- α was observed at 20 μ M DIM (18 \pm 0.1 fold of induction) and 40 μ M (4.7 \pm 0.4 fold of induction). The 60 μ M DIM treatment was the most effective concentration to suppress the relative expression of MCP-1, IL-6 and TNF- α by 84.3%, 90% and 40%, respectively. Proinflammatory protein production and its relative gene expression presented a decreasing similar pattern as an inverse function of DIM concentration. These results indicate that the treatment with DIM reduced the inflammatory response induced in the co-cultures.

3.2. DIM Increases Cytoplasmic IRS-1 pY612 and Akt-1/PKB pT308 Concentration

To examine the effect of DIM on molecules of the insulin signaling pathway, intracellular proteins isolated from lysates of adipocytes co-cultured with RAW264.7 macrophages were immunoblotted with antibodies against IRS-1pY612 and

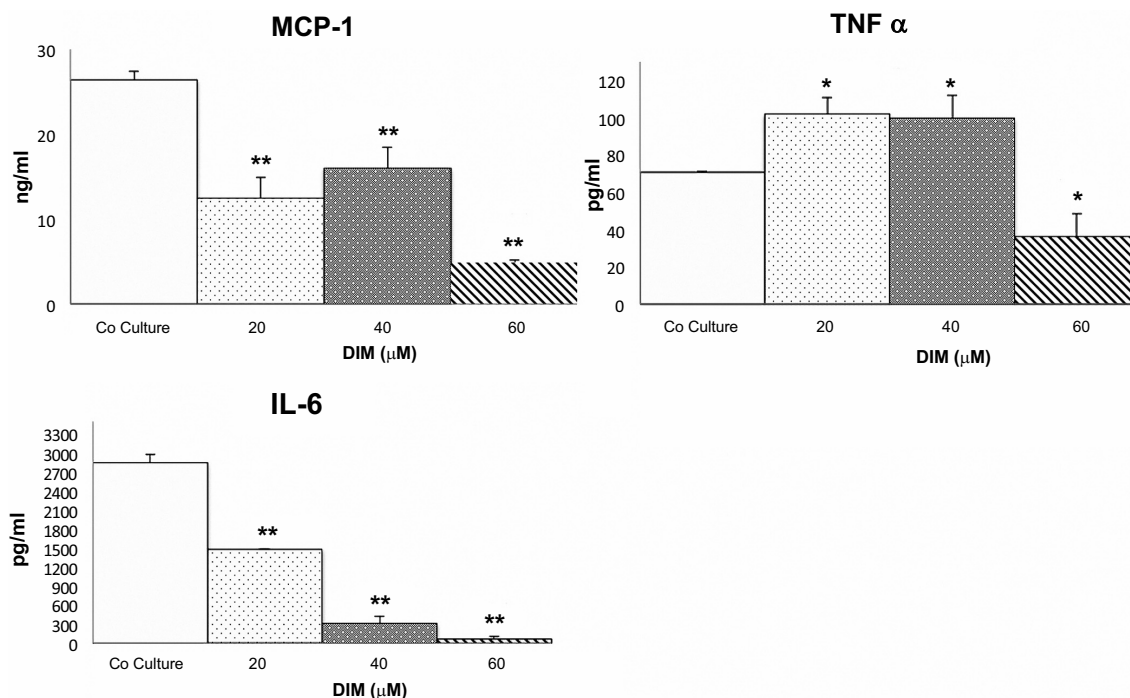


Fig. (1). 3-3-diindolylmethane (DIM) effect on MCP-1, IL-6 and TNF- α production in supernatants of 3T3-L1 adipocytes (1×10^5) co-cultured with RAW 264.7 macrophages (5×10^4) in a transwell system with a 0.4 μm porous membrane, treated with 20 μM , 40 μM , and 60 μM DIM during 24 h followed by 100 nM insulin for 20 min. Determinations were carried out in triplicate using individual enzyme-linked immunosorbent assay and expressed as the mean value \pm SD * $p < 0.01$, ** $p < 0.001$.

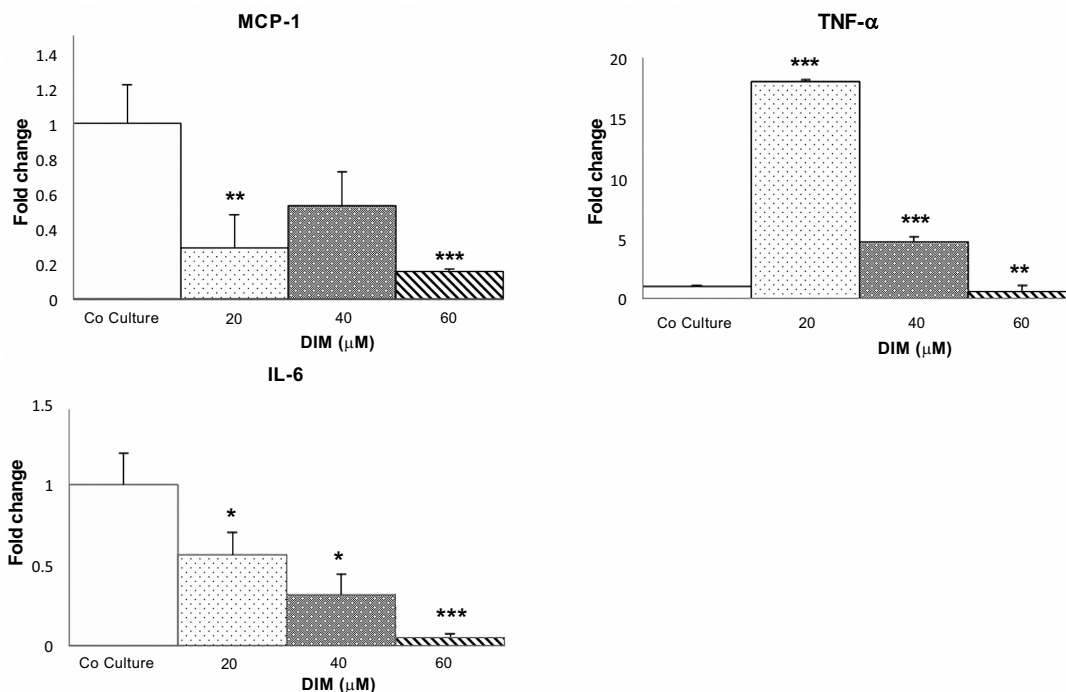


Fig. (2). 3-3-diindolylmethane (DIM) effect on MCP-1, IL-6, and TNF- α mRNA relative expression of 3T3-L1 adipocytes (1×10^5) co-cultured with RAW264.7 macrophages (5×10^4) in a transwell system with a 0.4 μm porous membrane treated with 20 μM , 40 μM , and 60 μM DIM during 24 h, followed by 100 nM insulin for 20 min. Determinations were performed by qPCR. Results were normalized based on the expression of actin- β gene and analyzed by the comparative relative expression $CT (2^{-\Delta\Delta C_T})$ method [31] * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Akt-1/PKB pT308. Both IRS-1pY612 and Akt-1/PKBpT308 proteins have been described as critical phosphorylated forms in the insulin-dependent translocation of GLUT-4 [32, 33]. As shown in Fig. (3), concentration of IRS-1pY612 and

Akt-1/PKB pT308 increased in a DIM concentration dependent manner, inversely correlating with the decreasing response of the relative expression and production of inflammatory proteins. IRS-1pY612 concentration was higher

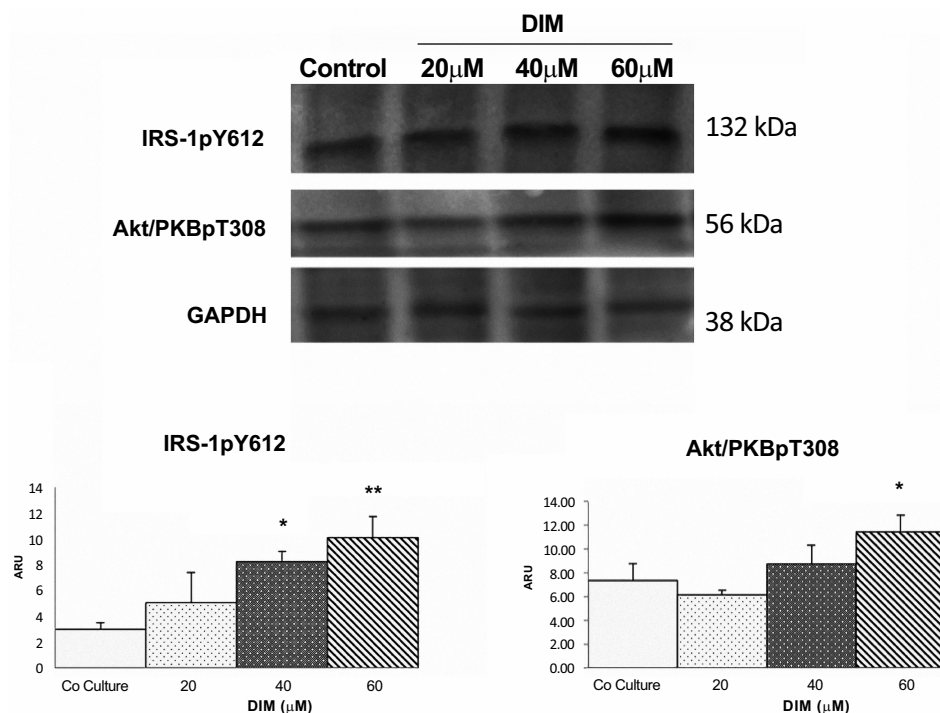


Fig. (3). 3-3-diindolylmethane (DIM) effect on IRS-1 pY612 and Akt/PKB pT308 proteins production from 3T3-L1 adipocytes (1×10^5) co-cultured with RAW 264.7 macrophages (5×10^4) in a transwell system with a 0.4 μm porous membrane, treated 20 μM , 40 μM , and 60 μM of DIM during 24 h, followed by 100 nM insulin for 20 min. Determinations were performed in triplicate using Western Blot analysis. Each bar represents the mean value \pm SD. ARU, Area Relative Units * $p < 0.01$; ** $p < 0.001$.

after exposure to 20 μM DIM ($1.68 \text{ X} \pm 0.5$ fold change), 40 μM ($2.75 \text{ X} \pm 0.1$ fold change; $p < 0.01$), and 60 μM ($3.4 \text{ X} \pm 0.1$ fold change; $p < 0.001$), compared with the control. Akt-1/PKB pT308 concentration also increased after exposure to 40 μM DIM ($1.2 \text{ X} \pm 0.2$ fold change) and 60 μM ($1.6 \text{ X} \pm 0.1$ fold change; $p < 0.01$), providing evidence that DIM could positively improve the metabolic pathway for GLUT-4 translocation.

4. DISCUSSION

Vegetables of the *Brassica* genus are an abundant source of dietary phytochemicals with a high content of glucosinolates, precursors of DIM, that have gained scientific interest in the last years due to their multiple documented health benefits [3].

Obesity is a complex metabolic condition associated to a subclinical chronic inflammation resultant from the interaction of adipocytes and adipose tissue infiltrating macrophages [34, 35]. Proinflammatory molecules promote degradation of IRS-1, deleteriously affecting glucose metabolism, leading to IR [27, 28]. In the current study we evaluated the effectiveness of 20 μM DIM, 40 μM and 60 μM to improve intracellular IRS-1 pY612 and Akt-1/PKB pT308 concentration, and the associated reduction of pro-inflammatory cytokines production in an *in vitro* model of obesity-induced inflammation constituted by the indirect co-culture of adipocytes and macrophages.

Our results show the most effective concentration of DIM to exert an inverse concentration-dependent inhibitory

effect on MCP-1, IL-6 and TNF- α production and relative expression was 60 μM , indicating that downregulation occurs through reduction of the corresponding mRNA. This antiinflammatory activity of DIM through the inhibition of the production of TNF- α and IL-6 has also been described in a lipopolysaccharide-induced inflammation model in murine macrophages [9]. Likewise, significant reduction of IL-6 was reported in a primary murine co-culture of adipocytes and macrophages [7]. Notwithstanding, the reducing effect of DIM on MCP-1 production and relative expression shown in our study, has not been reported previously. This finding is relevant since MCP-1 plays an important role in the establishment of the proinflammatory profile of obesity, promoting macrophages adipose-tissue migration [36].

Interestingly, an increase in TNF- α production was observed when co-cultures were exposed to 20 μM or 40 μM DIM. This phenomenon could be attributed to a paradoxical effect of DIM at concentrations below 60 μM . A paradoxical behaviour has also been described in other potent anti-inflammatory mediators, like IL-10, capable to inhibiting cytokine releasing in most cell types, but acting as a pro-inflammatory cytokine under certain circumstances [37, 38] However, accomplishment of pharmacokinetic studies is necessary in order to further understand DIM antiinflammatory effect in this model.

An induction pattern of IRS-1 pY612 and Akt-1/PKB pT308 was exerted by DIM, showing a concentration-dependent response. This finding could probably explain, at least in part, the improvement of glucose metabolism [7, 16] and the glucose and insulin reduction in obese mice with

diabetes mellitus type 2 [19] attributed to I3C. Specific phosphorylation of IRS-1 pY612 and Akt-1/PKB pT308 constitutes key downstream steps on the insulin signaling pathway leading to glucose uptake, accounting up to 80% of glucose transporter 4 (GLUT-4) translocation [32, 39, 40]. When IRS-1 pY612 decreases, glucose uptake induced by insulin is prevented promoting IR [37-39]. TNF- α interferes signaling pathway through phosphorylation of IRS-1 in serine residues, inducing IRS-1 degradation, uncoupling the insulin receptor, and interrupting receptor autophosphorylation [27, 36, 41-43]. Correspondingly, IL-6 inhibits insulin receptor autophosphorylation, blocking IRS-1 binding to the receptor through the activation of STAT1 and SOCS3 [21, 27]. Hence, the increased intracellular IRS-1 pY612 and Akt-1/PKB pT308 concentration observed in our study could probably be attributed to reduction of MCP-1, IL-6 and TNF- α production. This is the first report showing the beneficial effects of DIM on IRS-1 pY612, Akt-1/PKB pT308 and MCP-1 altogether in an indirect co-culture of adipocytes and macrophages.

CONCLUSION

We conclude that DIM is capable to promote phosphorylation of molecules involved in the insulin signaling transduction pathway and improve the inflammatory profile in this *in vitro* model of obesity-induced inflammation. Our findings suggest that DIM could probably facilitate GLUT-4 translocation and glucose uptake; however, the evaluation of other molecules involved in these processes would provide more evidences regarding the potential therapeutic benefits of DIM in the treatment of metabolic abnormalities related to obesity and IR.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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ance, García-Bañuelos and Bastidas-Ramírez wrote the article and contributed equally as corresponding authors.

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