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Research Article

Global No-Flow Ischemia and Chemical Stress Condition Effects on D-3-hydroxybutyrate and Glucose Utilization in the Isolated Perfused Heart

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Abstract

The heart utilizes ketone bodies in preference to fatty acids and glucose under normal condition. This study was carried out to investigate the effect of global no-flow ischemia and chemical stress condition on the utilization of D-3-hydroxybutyrate (D-3-HB) and glucose with reference to the AMP-activated protein kinase (AMPK) and p38 mitogen activated protein kinase (p38 MAPK) signaling cascades in the isolated perfused heart. 2,4-dinitrophenol (DNP) and anisomycin (Aniso) are used to mimic hypoxia and ischemia respectively. DNP stimulated D-3-HB and glucose utilization. D-3-HB decreased the DNP-and Insulin-stimulated glucose utilization. The stimulatory effects of Insulin and DNP on glucose utilization were additive. Insulin and DNP enhanced D-3-HB utilization and the stimulatory effects of Insulin and DNP were not additive. Global no-flow ischemia-reperfusion, ionomycin (Iono) an activator of Ca^{2+}-calmodulin-dependent protein kinase kinase (CaMKK) and anisomycin an activator of p38 MAPK stimulate the utilization of D-3-HB. STO-609 and PD-169316 are a selective inhibitor of CaMKK and p38 MAPK respectively. STO-609 and PD-169316 abolished the increase in D-3-HB utilization in response to ionomycin, anisomycin, and global no-flow ischemia-reperfusion. We conclude that these results indicate the involvement of AMPK and p38 MAPK in the regulation of D-3-HB and glucose utilization during stress, and global no-flow ischemia. Stimulatory effect on D-3-HB utilization could be mediated via the AMPK and p38 MAPK signaling cascades.

Keywords: *D-3-hydroxybutyrate; Glucose; AMPK; p38 MAPK; Cardiac Metabolism; Ischemia*

Abbreviations

DL-3-HB: DL-3-Hydroxybutyrate; D-3-HB: D-3-Hydroxybutyrate; AMPK: AMP-Activated Protein Kinase; p38 MAPK: p38 Mitogen Activated Protein Kinase; DNP: 2,4-Dinitrophenol; Aniso: Anisomycin; Ara-A: Adenine 9-β-D-Arabinofuranoside; Itub: 5-Iodotubercidin; STO-609: 7-Oxo- 7-H- benzimidazo [2.1-] benz [de] isoquinoline-3- carboxylic acid - acetic acid; Iono: Ionomycin; DSMO: Dimethyl Sulfoxide; MKHM: Modified Krebs-Henseleit Medium; ICAR: Aminoimidazole-4-Carboxamide Ribonucleotide; CaMKK: Ca^{2+} Calmodulin-Dependent Protein Kinase Kinase; PI3K: Phosphatidylinositol 3-Kinase; GLUT4: Glucose Transporter Type 4; PFK-2: Phosphofructokinase-2; PDH: Pyruvate Dehydrogenase; AMPKK (LKB1): AMP-Activated Protein Kinase Kinase

Introduction

The heart is a remarkably flexible to adapt substrates to meet its energy demand under physiological and non-physiological conditions. The heart preferentially utilizes long-chain fatty acids as a main source of energy (40% - 60%), and glucose, lactate, D-3-hydroxybutyrate (D-3-HB), acetoacetate and amino acids contribute 20% to 40% [1]. Metabolic remodeling of myocardial energy substrates during heart failure manifests metabolic shifts such as, a decrease in fatty acid oxidation, an increase in glycolytic pathway, a decrease in glucose oxidation, and an increase of ketone bodies oxidation [2-5]. It is well known that the myocardium utilizes glucose and D-3-HB as a source of energy. They have a vital role in fulfilling the heart high energy demand under physiological and pathological conditions. Ketone bodies compete with glucose and fatty acids for utilization by the heart [6,7]. Fatty acids and glucose metabolism in the heart is mediated by AMP-activated protein kinase (AMPK) signaling cascade. AMPK activation increases glucose and fatty acid oxidation and inhibits protein

synthesis in the heart [8-12]. The stimulation of AMPK enhances catabolic pathways to provide the heart with ATP and restricts the consumption of ATP [13]. Maintaining cellular ATP levels is vital for the heart viability and function under stress related conditions such as, ischemia, ischemia-reperfusion, hypoxia, and anoxia [8,13,14]. Hypoxia and ischemia activate p38 mitogen activated protein kinase (p38 MAPK), which has a role in cardiac metabolic regulation [10,15,16]. D-3-HB other than being a fuel substrate for the heart, it has a regulatory role in metabolism and other cardioprotective effects [17-19] and therapeutic implications. In this study we investigate the utilization of D-3-HB as a sole substrate and in competition with glucose. Also, the role of AMPK and p38 MAPK signaling cascades in D-3-HB and glucose utilization under chemical stress and global no-flow ischemia-reperfusion in the isolated perfused rat heart. The modulation of AMPK and p38 MAPK activation was achieved by using specific activators and inhibitors of both signaling cascades. To mimic hypoxia, and ischemia-reperfusion, we used 2,4-dinitrophenol (DNP), and anisomycin (Aniso) as chemical stressors respectively.

Materials and Methods

Material

Animals

Normal male Albino Wister rats weighing between 250 - 350g. Animals were housed under a constant room temperature of 22°C and a controlled light cycle (lights on between 06.00 - 18.00h). The rats had free access to food and water. The diet consisted of pellets containing 13% protein and 3% fat, manufactured by Grains and Flour Mills Organization, Jeddah, Saudi Arabia. All experiments were conducted in accordance with the guidelines of Umm Al-Qura University Council of Animal Care and were approved by the animal care committee of Umm Al-Qura Research Institute.

Chemicals

DL-3-hydroxybutyrate (sodium salt) (DL-3-HB), nicotinamide adenine dinucleotide (disodium salt, oxidized and reduced forms), D-3-hydroxybutyrate dehydrogenase (EC.1.1.1.30), acetoacetate (lithium salt), 2,4-dinitrophenol (DNP), adenine 9-β-D-arabinofuranoside (Ara-A), 5-iodotubercidin (Itub), 7-Oxo- 7-H- benzimidazo [2.1-] benz [de] isoquinoline-3- carboxylic acid - acetic acid (STO-609), ionomycin (Iono), anisomycin (Aniso), and dimethyl sulfoxide (DMSO) as vehicle (v). all other chemicals maintained the highest available quality and were obtained from SIGM-ALDRICH, USA. D-Glucose (ARISTAR), and bovine Insulin were obtained from BDH chemicals pls.U.K. Insulin stock solution was prepared by dissolving crystalline bovine Insulin in 0.9% NaCl solution containing 0.01 N HCl; aliquots were stored at -20°C.

Methods

Media

The saline medium consisted of 142.2 mM sodium chloride and 0.5 mM sodium bicarbonate, and when equilibrated with atmospheric CO_2 at 4°C, had a pH of 7.4. The saline medium was used during the preparation of cannulation to cool the heart following excision. Hearts were perfused for either two hours or 90 minutes with Krebs-Henseleit medium modified to contain half of the concentration of calcium and magnesium (MKHM) and oxygenated by equilibration with 5% CO_2 in oxygen. DL-3-HB was included at 5 mM corresponding to an initial concentration of the metabolically active D-3-HB of 2.5 mM. When the vehicle (v), DMSO was used, its concentration was no more than 0.2% v:v and the addition of other substances are shown in the perfusion method section.

Perfusion method

We used the Fisher and O'Brien and Sultan [17] non-working heart perfusion technique to perfuse the rat heart. This technique involves the continuous infusion of fresh media into a volume of recirculating perfusate that is kept constant by balanced withdrawal. Hearts were removed from fed rats under light diethyl ether anesthesia and placed in a cooled saline medium at 4°C and prepared for cannulation. Hearts were perfused with MKHM containing substance(s) as shown in the text and figure legends at a pressure of 40 mmHg, and at an infusion rate of 30.7 ± 0.1 $ml.hr^{-1}$ (122) Group 11 to 31 (Figure 3 to 7) and 30.6 ± 0.1 $ml.h^{-1}$ (58) group 1 to 10 (Figure 1 and 2), for 90 or 120-minutes respectively. The perfusate temperature was 37°C. The perfusate was passed through a 47 mm Millipore disc of 0.47 µm pore diameter (Millipore Corporation, Bedford, Mass, USA) supported by a Whatman No. 54 paper filter. Samples of perfusate were collected for five minutes and alternate samples were analyzed. We collected twelve or six samples during the 2nd hour or the last 30 minutes of the perfusion period for analytical purposes, six or three alternate fractions were used to determine the D-3-HB, acetoacetate, glucose, and lactate concentrations. Coronary flow of each perfused heart was measured at the 3rd minute 57.57 ± 1.33 (118) [ml. (g. dry $wt)^{-1}.min^{-1}$] and at the end of the perfusion period 60.1 ± 1 (122) [ml. (g. dry $wt)^{-1}.min^{-1}$]. Group 11 to 31 (Figure 3 to 7) and 64.2 ± 1.5 (58) [ml. (g. dry $wt)^{-1}.min^{-1}$]for group 1 to 10 (Figure 1 and 2). The heart rate, vigor, and rhythm were noted periodically, and the mechanical performance and metabolic stability were used as criteria of a successful preparation [17]. Throughout the work the concentrations of

substances when used were as follow: Glucose (5 mM), DL-3-HB (5 mm), DNP (50 µM), Insulin (2 $mU.ml^{-1}$), Ara-A (1 mM), Itub (5 µM), Iono (1.3 µM), STO-609 (2.5 µM), Aniso (10 µM) and PD-169316 (1 µM).

Global no-flow ischemia protocol

Three groups of hearts19, 20 and 21 were perfused with MKHM, containing DL-3-HB (5 mM) and vehicle in the absence and presence of either PD-169316 (1 µM) or STO-609 (2.5 µM) for 10 minutes. This served as the equilibration period of aerobic perfusion as coronary flow reached 60 ± 1.61 [ml. (g. dry wt)$^{-1}$.min^{-1}] followed by 15 minutes of global no-flow ischemia. Then the perfusate flow through the heart is completely interrupted resulting in a subsequent 65-minute period of aerobic reperfusion with MKHM containing DL-3-BH (5 mM) in the absence and presence of PD-169316 or STO-609. The coronary flow at the end of the reperfusion period was 63.71 ± 2 [ml. (g. dry wt)$^{-1}$.min^{-1}]. Heart beats ceased between 2 - 4 minutes after stopping the flow of the perfusate and restarted after 1 - 2 minutes of reperfusion. On examining the heart at the end of perfusion period, the heart was soft and there were no apparent ischemic patches.

Analytical methods

The estimation of D-3-HB, acetoacetate, and lactate were performed by the methods of Williamson and Mellanby [20], Mellanby and Williamson [21] and Gutmann and Wahlefeld [22] respectively. Glucose was determined by hexokinase method, using COBAS INTEGRA 400 Analyzer.

Calculation and expression of the results

In the steady state condition, the concentrations of the substrate in successive fractions were not measurably different. The second hour of the perfusion period represents the steady state period. During this period the following equation was used to estimate the rate of glucose, and D-3-HB utilization, and the rate of lactate, and acetoacetate production:

$u = i(a-x)/w$

Where u: the rate of utilization (negative if production), [µmoles. (g. dry wt)$^{-1}$.h^{-1}]

i: The rate of infusion, $ml.h^{-1}$

a: The initial perfusate concentration, mM

x: The perfusate concentration, mM

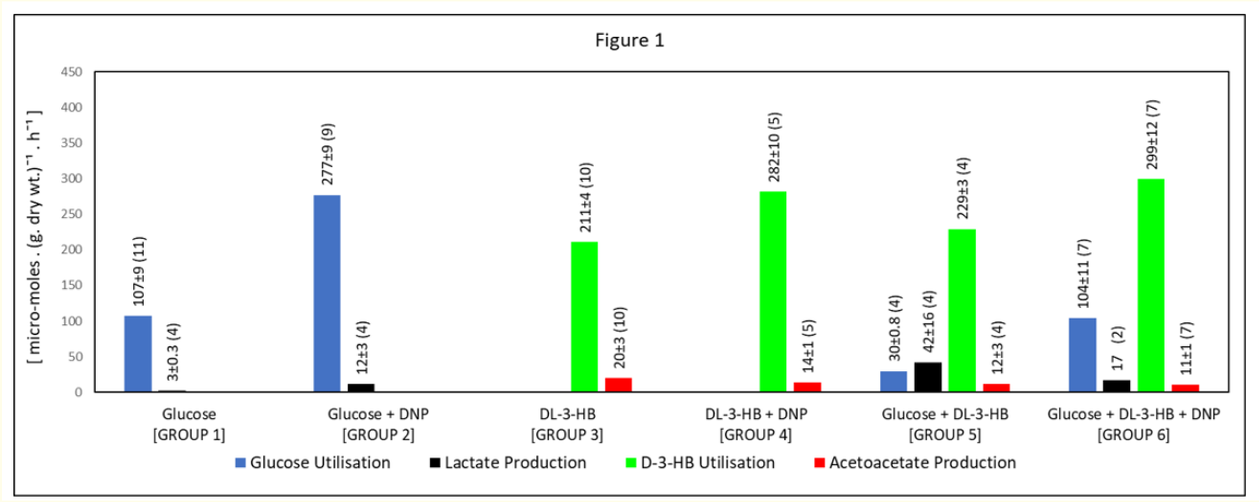
w: The dry weight of the heart, g.

The difference between the rate of D-3-HB utilization and the rate of acetoacetate production is assumed to be the rate of D-3-HB oxidation. Results were expressed as a mean of individual experiments ± SEM, and the number of observations given in parentheses. Comparisons between groups (11 to 31) were assessed by the two-tailed Student's t-test for independent observations using Texasoft, WINKS SDA Software, 6th Edition, Cedar Hill, Texas, 2007. Comparisons between groups (1 to 10) were assessed by the two-tailed Student's t-test for independent observations using excel spread sheet built-in program. The normality of the experimentally measured data was assessed using the Normal Probability Plot technique. All measured data were deemed to be approximately normally distributed for each group. When two sets of data are compared, an F-test was used to test if the variance of the two groups are statistically equal by assessing whether or not there is not enough evidence within the data to reject the null hypothesis that the two group variance are equal at the 0.05 significance level. Furthermore, a t-test (t-Test: Two Sample Assuming Equal Variances or t-Test: Two Sample Assuming Unequal Variances) was used to test if there is a significant difference (significance level of alpha < 0.05) between the mean of two groups and whether the data provide strong enough evidence to reject the null hypothesis to conclude the two population means are different.

Results

The effect of DNP on glucose utilization in the absence and presence of insulin

The maximum effect of DNP on D-3-HB utilization was observed at 50 µM without any deterioration effect on the heart preparation, whereas DNP at concentration 100 µM the heart beats were irregular, and the preparation was unstable [23]. As shown in figure 1, DNP stimulated glucose utilization by 2.6-fold $p \leq 0.001$ (group 1 vs 2), when glucose is the sole exogenous substrate. Lactate production was no greater than 1 and 2% of glucose utilization in the absence and presence of DNP respectively. Insulin stimulated glucose utilization by 2.8-fold $p \leq 0.001$ (group 1 vs 7), lactate production accounts for 14% of the utilized glucose. DNP increased the glucose utilization by 1.6-fold $p \leq 0.01$ (group 7 vs 8) in the presence of Insulin, and 8% of the utilized glucose recovered as lactate. There is an additive stimulatory effect of DNP and Insulin (group 1 vs 8) Insulin and DNP combined effect increased the glucose utilization by 4.6-fold, and about 8% of the utilized glucose recovered as lactate.



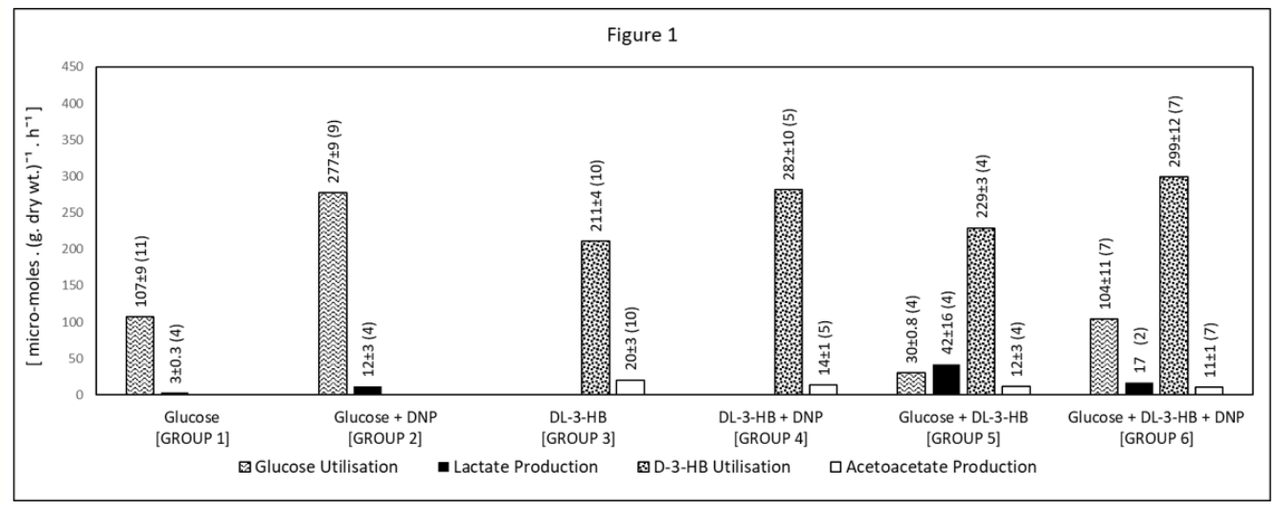


Figure 1: *The effect of DNP on glucose and D-3-HB utilization. Hearts were perfused with MKHM containing glucose (5 mM) (group 1), glucose + DNP (group 2), DL-3-HB (group 3), DL-3-HB + DNP (group 4), glucose + DL-3-HB (group 5), and glucose + DL-3-HB + DNP (group 6) for 120 minutes except (group 3) is perfused for 90 minutes as described in the method section. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rates of utilization and production were estimated during the last 60 minutes of perfusion period.*

The effect of DNP on D-3-HB utilization in the absence of insulin

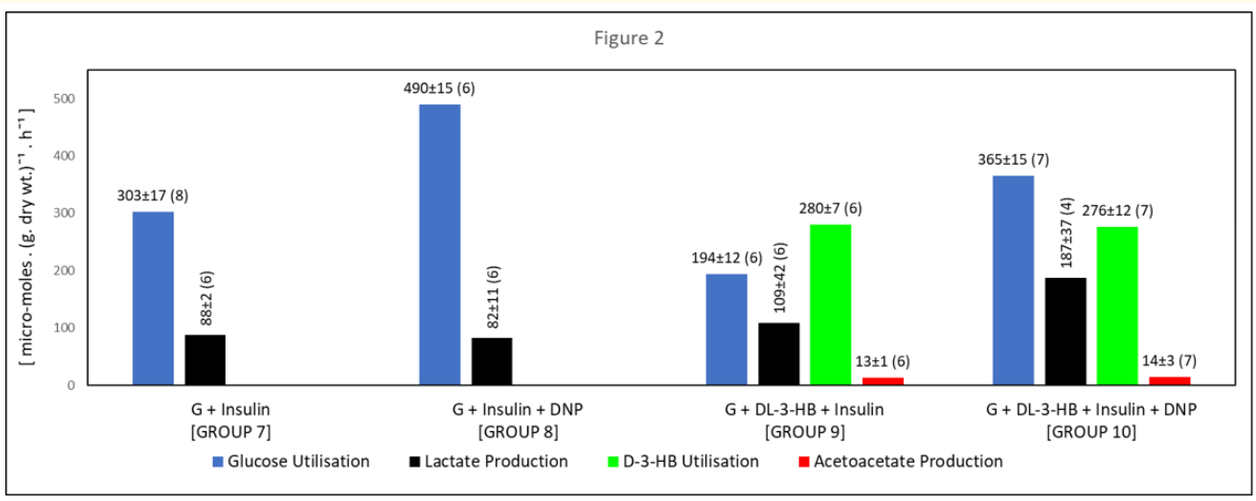
The DNP increased the rate of D-3-HB utilization by 1.4-fold $p \leq 0.001$ (group 3 vs 4) (Figure 1) when D-3-HB is the sole exogenous substrate acetoacetate production were 9 and 5% of D-3-HB utilization in the absence and presence of DNP respectively.

The effect of DNP on glucose and D-3-HB utilization in the absence of insulin

As shown in figure 1 when both substrates (glucose and DL-3-HB) are included in the medium, D-3-HB and glucose are utilized by the heart. D-3-HB inhibited glucose utilization by 72% $p \leq 0.01$ (group 5 vs 1) (Figure 1) and about 70% of the utilized glucose recovered as lactate. DNP enhanced D-3-HB utilization by 31% $p \leq 0.01$ (group 5 vs 6) (Figure 1) without any significant effect on acetoacetate production. D-3-HB inhibited DNP-stimulated glucose utilization by 62% $p \leq 0.01$ (group 2 vs 6) (Figure 1). DNP restored the basal rate of glucose utilization (group 1 vs 6) (Figure 1) and DL-3-HB inhibited DNP-stimulated glucose utilization by 62% $p \leq 0.01$ (group 2 vs 6) (Figure 1) and 8% of the utilized glucose restored as lactate, acetoacetate production was about 4% of the utilized D-3-HB. Glucose has no significant effect on D-3-HB utilization.

The effect of DNP on glucose and D-3-HB utilization in the presence of insulin

In figure 2 groups 9 and 10 both exogenous substrates (glucose and DL-3-HB) are included in the medium. Both substrates are utilized, and DNP increased glucose utilization by 1.9-fold $p \leq 0.001$ (group 9 vs 10) (Figure 2) without any additive effect on D-3-HB utilization. Adding Insulin, or DNP, or (Insulin and DNP), increased glucose utilization by 2.8, 2.6 and 4.6-fold in groups 2, 7, and 8 respectively (Figure 1 and 2) indicating an additive effect of Insulin and DNP. DNP and Insulin enhanced D-3-HB utilization 34% $p \leq 0.01$ (group 5 vs 6) and 22% $p \leq 0.01$ (group 5 vs 9) (Figure 1 and 2) respectively. Combining DNP and Insulin has no significant additive effect on D-3-HB utilization, groups $p \leq 0.01$ (9 vs 10) and $p \leq 0.01$ (10 vs 6) (Figure 1 and 2).



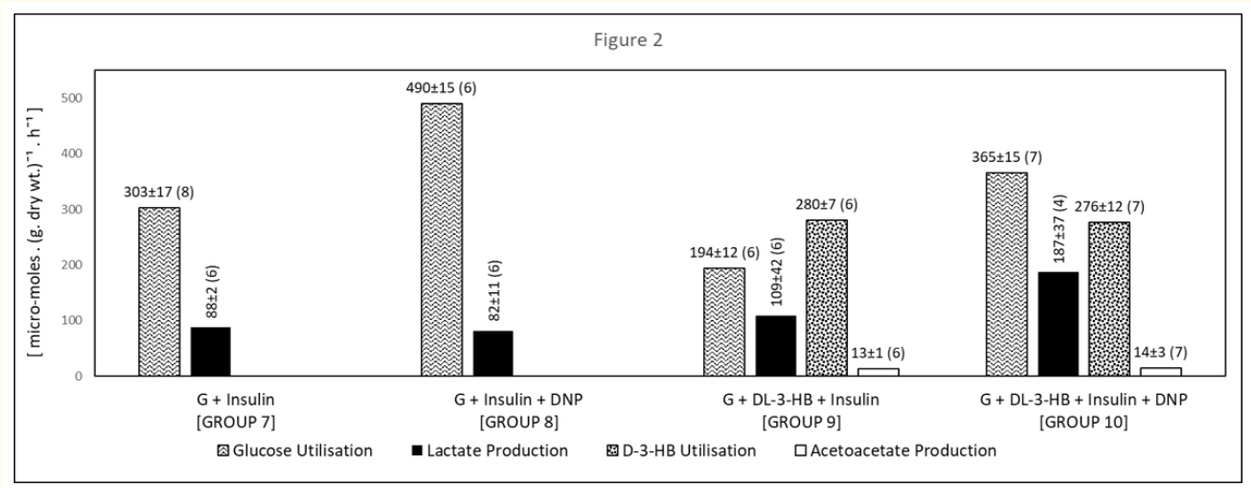


Figure 2: *The effect of DNP and Insulin on glucose and D-3-HB utilization. Hearts were perfused with MKHM containing glucose + Insulin (group 7), glucose + Insulin + DNP (group 8), glucose + DL-3-HB + Insulin (group 9) and glucose + DL-3-HB + Insulin + DNP (group 10), for 120 minutes as described in method section. Results indicate means ± SEM and the number of individual observations is given in parentheses. The rates of utilization and production were estimated during the 2nd 60 minutes of perfusion period.*

The effect of Insulin on glucose and D-3-HB utilization

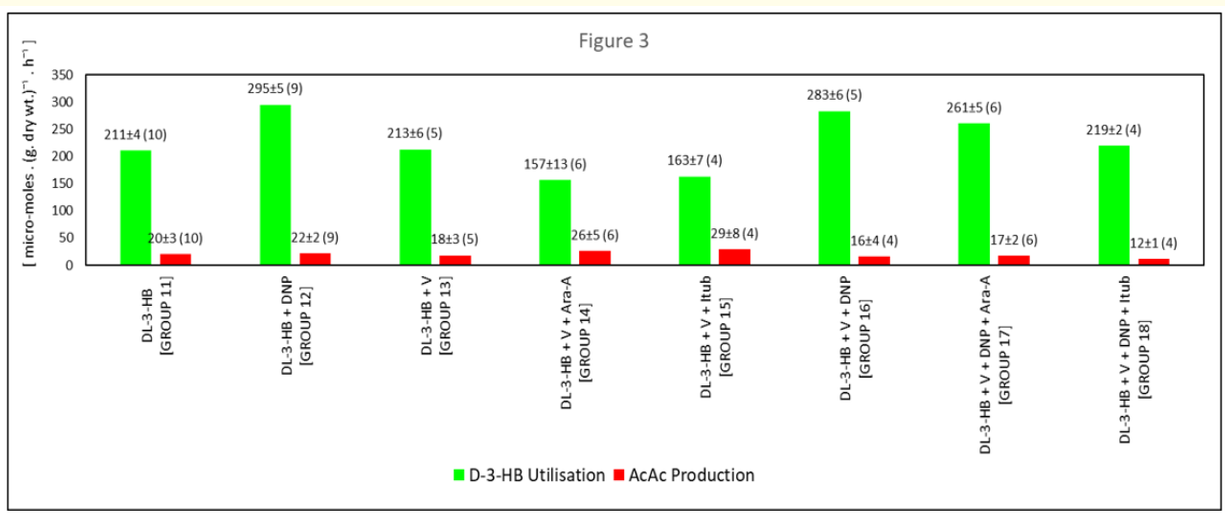
Insulin stimulated glucose utilization by 2.8-fold $p \leq 0.001$ (group 1 vs 7) (Figure 1 and 2), when glucose is the sole exogenous substrate, and 15% of the utilized glucose recovered as lactate. Insulin-stimulated glucose utilization is decreased by 36% $p \leq 0.001$ (group 7 vs 9) (Figure 2) in the presence of DL-3-HB and 28% of the utilized glucose recovered as lactate. Insulin and DNP has a combined stimulatory effect on glucose utilization, about 4.5-fold $p \leq 0.001$ (group 8 vs 1) (Figure 1 and 2) and 8% of the utilized glucose recovered as lactate, and the combined stimulatory effect of Insulin and DNP is decreased 25% $p \leq 0.001$ (group 8 vs 10) (Figure 2) in the presence of DL-3-HB. Insulin stimulated D-3-HB utilization by 22% in the absence and presence of DNP. The effect of DNP and Insulin on D-3-HB utilization are not additive.

The effect of DL-3-HB on glucose utilization

DL-3-HB inhibited glucose utilization by 72% $p \leq 0.001$ (group 1 vs 5) (Figure 1) and 70% of the utilized glucose recovered as lactate. DL-3-HB inhibited Insulin-stimulated glucose utilization by 36% $p \leq 0.001$ (group 9 vs 7) (Figure 2) and 28% of the utilized glucose recovered as lactate. DL-3-HB inhibited DNP-stimulated glucose utilization by 62% $p \leq 0.001$ (group 6 vs 2) (Figure 1) and 8% of the utilized glucose recovered as lactate. DL-3-HB inhibited (Insulin and DNP)-stimulated glucose utilization by 26% $p \leq 0.01$ (group 10 vs 8) (Figure 2) and 26% of the utilized glucose recovered as lactate.

The effect of DNP in the presence and absence of Ara-A and Itub on D-3-HB utilization

DNP activates AMPK in cardiomyocytes and Ara-A inhibited DNP-stimulated AMPK phosphorylation and glucose uptake [10]. Itub inhibited adenosine kinase in neonatal hearts [24], basal AMPKα_2 activity in skeletal muscle [25] and decrease aminoimidazole-4-carboxamide ribonucleotide (AICAR)-and cyanide-stimulated glucose uptake in the heart papillary muscle [11]. Figure 3 shows that DNP stimulated D-3-HB utilization by 40% $p \leq 0.001$ (group 11 vs 12) (Figure 3). The addition of vehicle dimethyl sulfoxide (DMSO) has no effect on basal D-3-HB utilization (group 11 vs 13) (Figure 3). Ara-A and Itub inhibited the basal rate of D-3-HB utilization by 26 and 23% $p \leq 0.005$ (group 14 vs 13) (Figure 3) and $p \leq 0.001$ (group 15 vs 13) respectively, without significant effect on acetoacetate production. DNP-stimulated D-3-HB partially inhibited by Ara-A 8% $p \leq 0.02$ (group 17 vs 16) (Figure 3) and abolished by Itub (group 16 vs 18) (Figure 3).



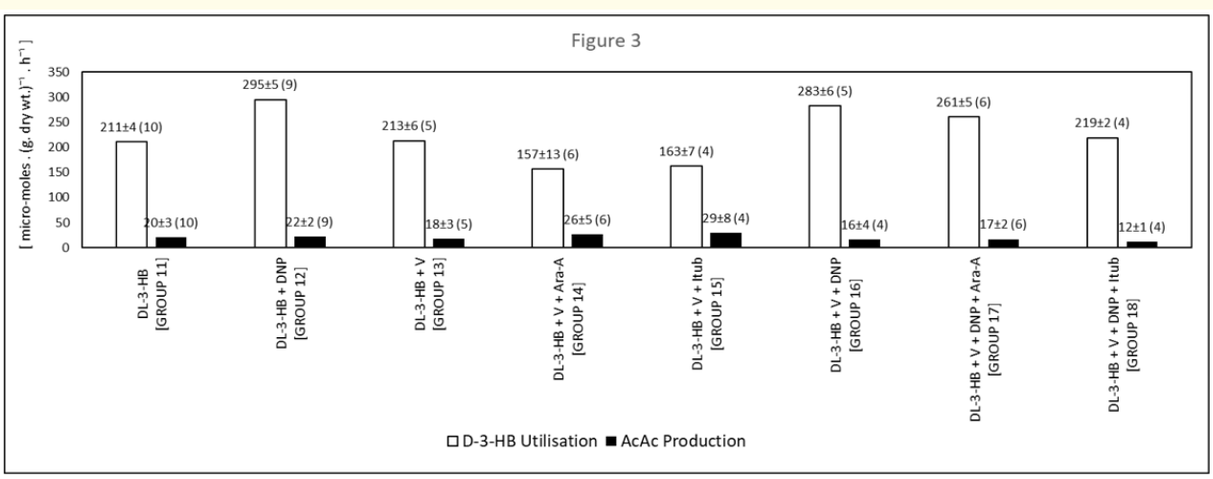
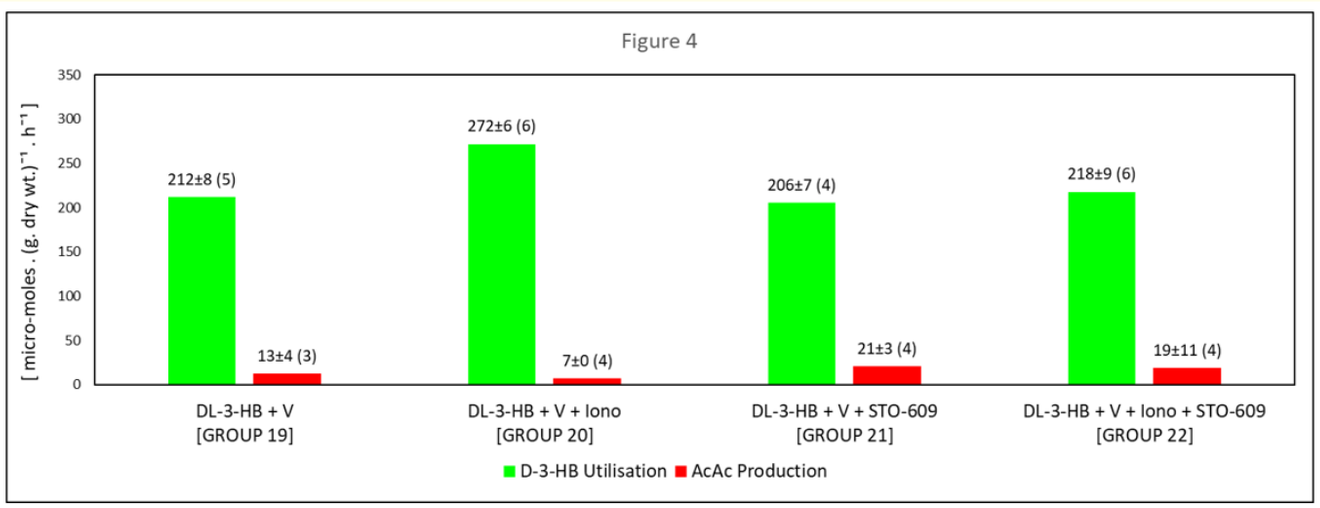


Figure 3: *The effect of DNP in the presence and absence of Ara-A and Itub on D-3-HB utilization. Hearts were perfused with MKHM containing DL-3-HB (group 11), DL-3-HB + DNP (group 12), DL-3-HB + vehicle (v) (group 13), DL-3-HB + v + Ara-A (group 14), DL-3-HB + v + Itub (group 15), DL-3-HB + v + DNP (group 16), DL-3-HB + v + DNP + Ara-A (group 17), and DL-3-HB + v + DNP + Itub (group 18) for 90 minutes as described in method section. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rate of D-3-HB utilization and acetoacetate production were estimated during the last 30 minutes of perfusion period.*

The effect of Ionomycin and STO-609 on D-3-HB utilization

The calcium ionophore, ionomycin, increased the cytosolic calcium concentration [26] and activated AMPK phosphorylation by the upstream kinase is Ca^{2+}calmodulin- dependent protein kinase kinase (CaMKK).This activation was inhibited by STO-609 [27-29] and DNP also induced an elevation of cytosolic Ca^{2+} [30-32]. We used ionomycin to activate AMPK through the upstream kinase, CaMKK. Figure 4 shows ionomycin stimulated the rate of D-3-HB utilization by 28% $p \leq 0.001$ (group 19 vs 20) (Figure 4) and almost all the utilized D-3-HB was fully oxidized. STO-609 abrogated the ionomycin-stimulated D-3-HB utilization and increased the rate of acetoacetate production to about 9% of the of the utilized D-3-HB $p \leq 0.005$ (group 22 vs 20) (Figure 4). STO-609 has no effect on the basal rate of D-3-HB utilization and acetoacetate production (group 21 vs 19) (Figure 4).



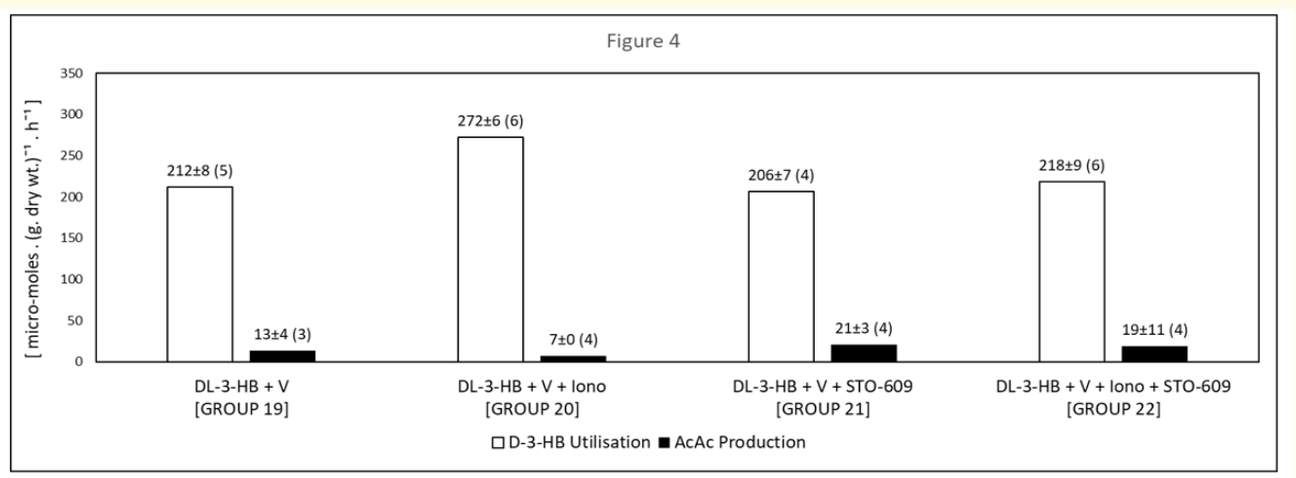
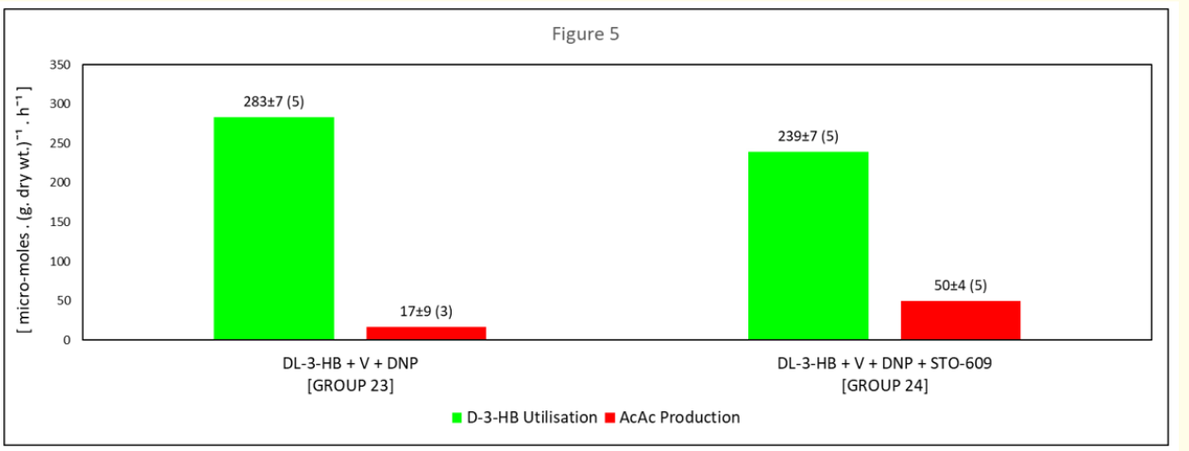


Figure 4: *The effect of Ionomycin and STO-609 on D-3-HB utilization. Hearts were perfused with MKHM containing DL-3-HB + vehicle (v) (group 19), DL-3-HB + v + Ionomycin (Iono) (group 20), DL-3-HB + v + STO-609 (group 21), DL-3-HB + v + Iono + STO-609 (group 22) for 90 minutes as described in method section. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rates of utilization and D-3-HB and acetoacetate production were estimated during the last 30 inutes of the perfusion period.*

The effect of DNP and STO-609 on D-3-HB utilization

Figure 5 shows that STO-609 partially inhibited DNP-stimulated D-3-HB utilization by 16% $p \leq 0.001$ (group 23 vs 24) (Figure 5) and the production of acetoacetate is increased to about 21% $p \leq 0.001$ (group 23 vs 24) (Figure 5).



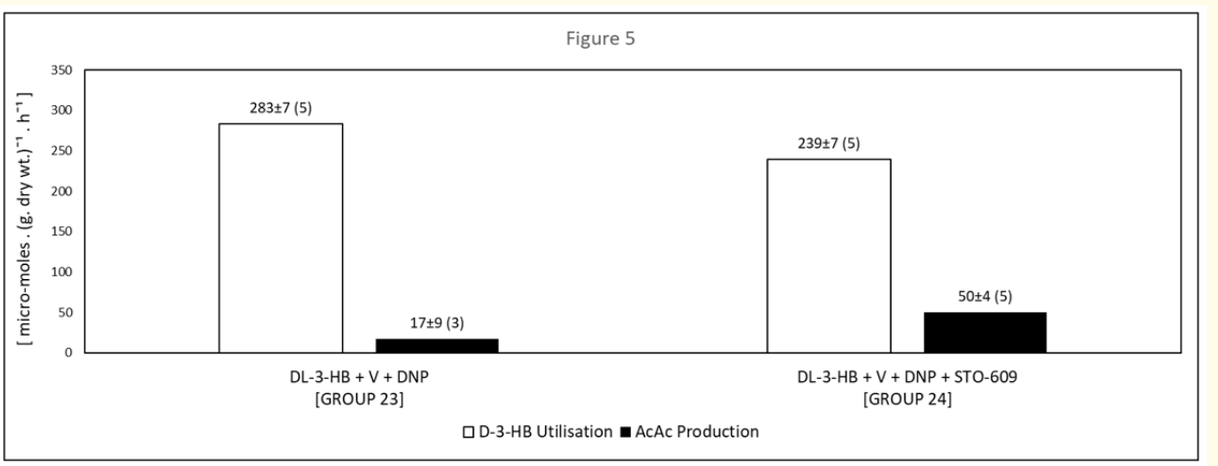
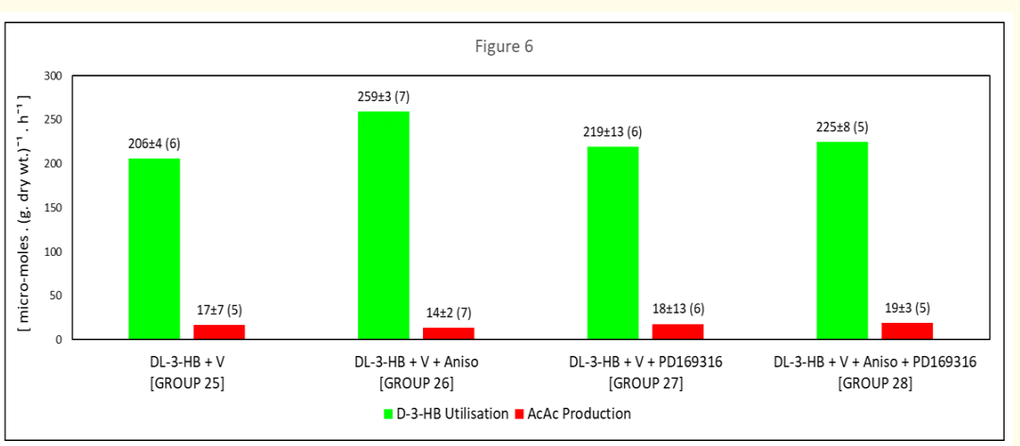


Figure 5: *The effect of DNP and STO-609 on D-3-HB utilization. Hearts were perfused with MKHM containing DL-3-HB + vehicle (v) + DNP (group 23) and DL-3-HB + v + DNP + STO-609 (group 24) for 90 minutes as described in method section. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rate of D-3-HB utilization and acetoacetate production were estimated during the last 30 minutes of perfusion period.*

The effect of Anisomycin and PD-169316 on D-3-HB utilization

Figure 6 shows anisomycin activated cardiac p38 MAPK [33,34]. DNP stimulated p38 MAPK activity and PD-169316 inhibited this effect in cardiomyocytes [10]. We used anisomycin to activate p38 MAPK which mimics the ischemia-reperfusion condition. Figure 6 shows that anisomycin stimulated the rate of D-3-HB utilization by 26% $p \leq 0.001$ (group 25 vs 26) (Figure 6) PD-169316 has no significant effect on basal D-3-HB utilization (group 27) (Figure 6) and PD-169316 fully abolished anisomycin-stimulated D-3-HB utilization (group 28) (Figure 6).



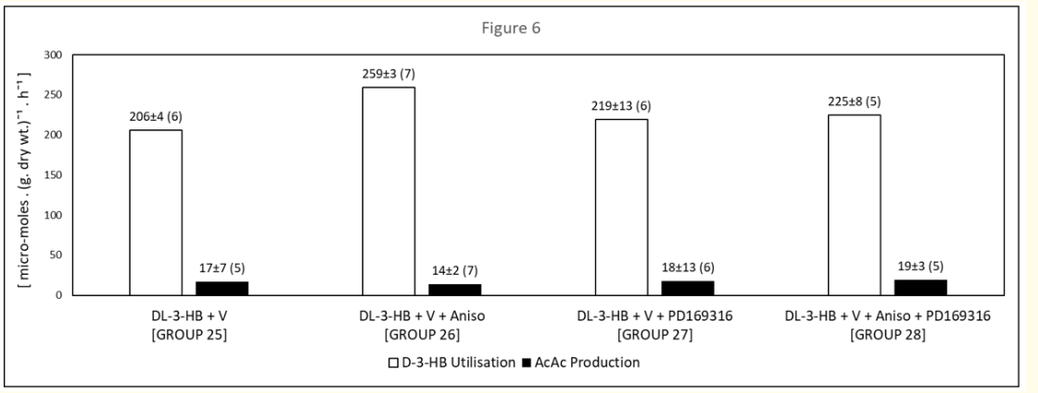
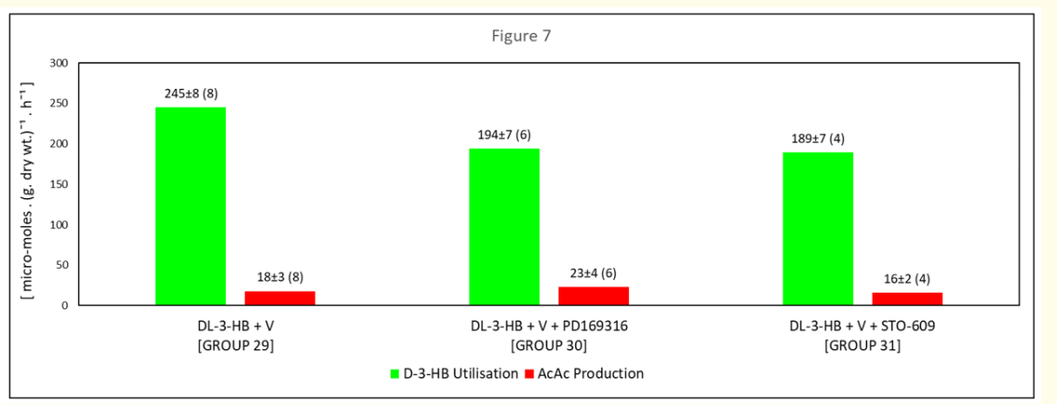


Figure 6: *The effect of Anisomycin and PD169316 on D-3-HB utilization. Hearts were perfused with MKHM containing DL-3-HB + vehicle (v) (group 25), DL-3-HB + v + Anisomycin (Aniso) (group 26), DL-3-HB + v + PD-169316 (group 27), and DL-3-HB + v + Aniso + PD-169316 (group 28) for 90 minutes as described in the method section. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rate of D-3-HB utilization and acetoacetate production were estimated during the last 30 minutes of perfusion period.*

The effect of global no-flow ischemia, PD-169316 and STO-609 on D-3-HB utilization

Hearts were subjected to no-flow ischemia for 15 minutes and allowed to equilibrate for 10 minutes, followed by a period of reperfusion for 65 minutes. The utilization of D-3-HB was estimated during the last 30 minutes of the reperfusion period. Comparing the rate of D-3-HB (group 25) (Figure 6) with (group 29) (Figure 7) the rate of D-3-HB utilization was increased by 19% $p \geq 0.001$ (group 29 vs 25) (Figure 6 and 7) during the reperfusion period. The rate of D-3-HB oxidation was 93% of the utilized D-3-HB. In another two groups of hearts subjected to no-flow ischemia, the addition of either PD-169316 (1 µM) (group 30) (Figure 7) or STO-609 (2.5 µM) (group 31) (Figure 7) to the media abolished the ischemia-reperfusion-stimulated utilization of D-3-HB.



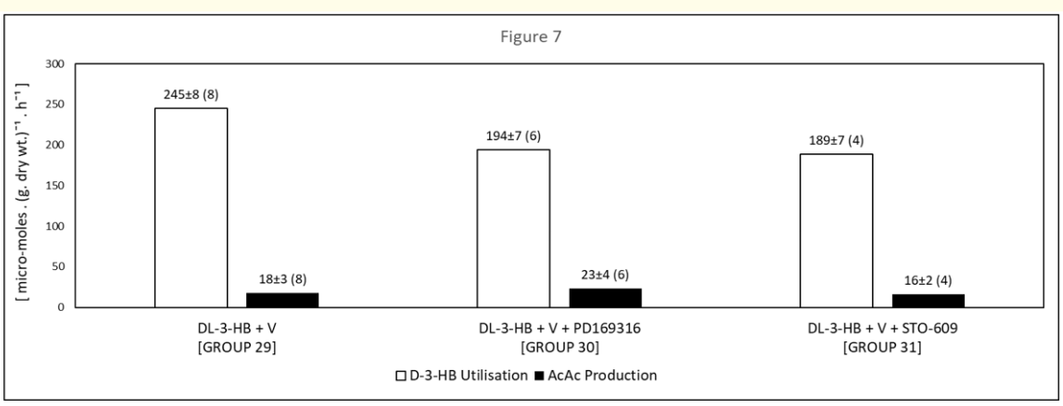


Figure 7: *The effect of no-flow ischemia-reperfusion in the absence and presence of PD-169316 and STO-609. Three groups of hearts 29, 30, and 31 were subjected to no-flow ischemia for 15 minutes as described in the method section, were perfused with MKHM containing DL-3-HB + vehicle (v) (group 19), DL-3-HB + v + PD-169316 (group 20) and DL-3-HB + v + STO-609 (group 21) for 65 minutes. Results indicate mean ± SEM and the number of individual observations is given in parentheses. The rate of D-3-HB utilization and acetoacetate production were estimated during the last 30 minutes of perfusion period.*

Discussion

Insulin and DNP enhanced glucose utilization by 2.8, and 2.6-folds respectively, and (Insulin and DNP)-stimulated effects on glucose utilization are additive 4.6-fold. This finding is consisting with others [10] in cardiomyocytes. The additive effect of Insulin and DNP is mediated by two different distinct signaling cascades, Insulin-stimulated glucose utilization via phosphatidylinositol 3-kinase (PI3K) which is responsible for translocation of GLUT-4 from cytosol to cellular plasma membrane and glucose transport, and DNP-enhanced glucose utilization is mediated by AMPK/p38 MAPK [6]. The activation of AMPK/p38 MAPK signaling cascade in cardiomyocyte is essential for DNP-stimulated uptake [6,10]. DNP-stimulated effect is mediated by increasing the cellular Ca^{+2} concentration [30,35]. AMPK enhanced phosphofructokinase-2 (PFK-2) and subsequent the glycolysis during ischemia [36]. Also increasing intracellular AMP/ATP ratio activates AMPK [37]. Ischemia and hypoxia evoke AMPK activation. There is more than one mechanism to enhance glucose utilization under stress conditions. D-3-HB inhibits glucose utilization and decreases Insulin- and DNP-stimulated glucose utilization. When both substrates glucose and D-3-HB are available, D-3-HB severely inhibits glucose utilization and 70% of the utilized glucose recovered as lactate, indicating glucose oxidation is inhibited, and preferential oxidation of D-3-HB by the heart under physiological condition. Introducing DNP to the medium to mimic hypoxia, DNP-stimulated glucose and D-3-HB utilization and D-3-HB inhibits DNP-stimulated glucose oxidation and 8% of the utilized glucose recovered as lactate. DNP raises cytosolic concentration of Ca^{+2} [30] which activates pyruvate dehydrogenase (PDH) leading to glucose oxidation [38,39]. D-3-HB inhibits Insulin-, DNP-, (Insulin and DNP)-stimulated glucose utilization by 36, 62 and 26% respectively. This variation of intense inhibition is due to the different mechanism of inhibiting glucose utilization by D-3-HB such as AMPK/p38 MAPK and protein kinase B (PKB) [6]. Glucose metabolism is a crucial source of energy during ischemia, and hypertrophy. D-3-HB oxidation could directly affect glucose oxidation by raising acetyl CoA, acetoacetyl CoA, and citrate. Acetyl CoA and citrate inhibits PDH, and PFK-2 respectively. DNP stimulates the utilization of D-3-HB in the isolated perfused heart in a concentration-dependent manner [23]. This stimulatory effect is due to either one or more of the multi effects of DNP. DNP is an uncoupler of the mitochondrial oxidative phosphorylation chain evokes mitochondrial membrane depolarization, a reduction in the mitochondrial membrane potential [40,41], a decrease in myocyte $NADH^{+}$ [32], a raise in the cytosolic concentration of Ca^{2+} and increases the AMP/ATP ratio [30,31,41]. DNP stimulates the activity of AMPK, p38 MAPK and the uptake of glucose in cardiomyocytes [10]. Either Ara-A or Itub decreased the basal rate of D-3-HB utilization, indicating a possible role for AMPK in normal conditions. Ara-A caused partial inhibition of the DNP-stimulated D-3-HB utilization, whereas Itub abolished the DNP-stimulated D-3-HB utilization indicating the role of AMPK in stressful conditions. The partial inhibition of Ara-A on DNP-stimulated D-3-HB utilization could be due to Ara-A inhibiting the AMPK α_2 isoform [42], which is also predominant in cardiac cells [43,44]. DNP activates both the α_1 and α_2 isoforms of AMPK [45], therefore, the DNP stimulatory effect could be a result of the activity from the AMPK α_1 isoform. The effect of Itub could be due to the inhibitory effect on adenosine kinase [24], adenosine transport [46], AMPK α_2 [25] and AMPKα_1 [47]. Ara-A partially inhibits DNP-stimulated AMPK and glucose uptake in cardiomyocytes [10] and Itub partially inhibits cyanide-stimulated glucose uptake in the hearts papillary muscle [11]. This is consistent with our findings, since Ara-A and Itub inhibit basal D-3-HB utilization.

The inhibition of basal D-3-HB utilization by both Ara-A and Itub (selective inhibitors of AMPKα_2), the partial and complete inhibition of DNP-stimulated D-3-HB utilization by Ara-A and Itub, respectively, and DNP's ability to raise the cytosolic Ca^{2+} concentration and the sensitivity of AMPKα_2 to AMP drove us to modulate the upstream kinase CaMKK. CaMKK activates AMPK [27,28,48], particularly AMPKα_1 [49], in response to the rise of cytosolic Ca^{2+} independently of the AMP/ATP ratio [50] and DNP increases cytosolic Ca^{2+} [30-32]. Ionomycin, is a CaMKK activator, stimulated D-3-HB utilization and was then abolished by STO-609, a selective inhibitor of CaMKK [29]. This supports the involvement of AMPK in regulating D-3-HB utilization, moreover; it indicates the role of calcium as an intracellular messenger in D-3-HB metabolism. STO-609 partially inhibited DNP-stimulated D-3-HB utilization, indicating that the DNP effect is partially mediated by increasing the level of cytosolic Ca^{2+}, further activating CaMKK. These findings support the possibility that the Ca^{2+}-CaMKK-AMPK signaling pathways mediates DNP-stimulated D-3-HB utilization.

DNP could also activate K_{ATP} channels by depolarizing the intra-mitochondrial membrane and altering the Ca^{2+} cytosolic concentration [51,52]. Pinacidil opens K_{ATP} channels and [53] has no significant effect on the basal rate of D-3-HB utilization [23]. Data do not support that enhancing K_{ATP} channels mediates DNP-stimulated D-3-HB utilization.

DNP activates p38 MAPK and AMPK in cardiomyocytes [10] and PD-169316 is a selective inhibitor of cardiac p38 MAPK [14,40]. Anisomycin potently activates cardiac p38 MAPK without stimulating the phosphorylation of AMPK and protein kinaseB (PKB) [33,54]. Anisomycin mimics ischemia-reperfusion in activating p38 MAPK [16,55,56]. Our finding that anisomycin stimulated D-3-HB utilization and that PD-169316 abolished this stimulation. This strongly supports the involvement of the p38 MAPK signaling pathway in the regulation of D-3-HB metabolism independent of the AMPK signaling pathway. We also demonstrated that PD-169316 had no significant effect on both basal D-3-HB utilization and DNP-stimulated D-3-HB utilization; although DNP activated p38 MAPK and PD-169316 partially

inhibited p38 MAPK in cardiomyocytes [10]. Therefore, we were unable to conclude that the DNP effect on D-3-HB utilization is mediated through the p38 MAPK signaling pathway, but did not exclude the involvement of p38 MAPK in D-3-HB regulation since anisomycin-activated D-3-HB utilization is abolished by PD-169316.

In this study, we demonstrated that modulating either the AMPK or p38 MAPK signaling pathways affects D-3-HB utilization. The stimulation and inhibition of the AMPK/p38 MAPK signaling pathways were associated with the stimulation and inhibition of D-3-HB utilization, respectively.

Mimicking either chemical hypoxia or ischemia-reperfusion is associated with the enhancement of the utilization of D-3-HB in the heart. Ischemia activates AMPK and p38 MAPK, and the activation of p38 MAPK occurs independently of AMPK [57]. PD-169316, an inhibitor of p38 MAPK, and STO-609, an inhibitor of CaMKK, cancel ischemia-reperfusion stimulated D-3-HB utilization. This supports the idea that ischemia-reperfusion-stimulated D-3-HB utilization is mediated by p38 MAPK and AMPK signaling cascades. Stress that alters the AMP/ATP ratio, mitochondrial and cytosolic concentration of calcium modulates D-3-HB metabolism. Ionomycin and anisomycin modulate AMPK and p38 MAPK, respectively, without affecting the AMP/ATP ratio, and both modulators stimulate D-3-HB utilization.

Pelletier and Coderre [10] reported that prolonged pretreatment of cardiomyocytes for 16h with D-3-HB caused a partial inhibition of DNP-stimulated AMPK and abolished DNP-stimulated p38 MAPK. D-3-HB partially inhibited DNP-stimulated glucose uptake in cardiomyocytes. They suggested that the inhibitory effect of D-3-HB on glucose uptake is due to the inhibitory effect of D-3-HB on AMPK/p38 MAPK activities. It is possible that D-3-HB can limit its own utilization under certain conditions by inhibiting AMPK/p38 MAPK activities, serving as a feed-back control. However, we did not estimate AMPK/p38 MAPK activities, and the perfusion period did not exceed 2 hours, therefore, we would not expect to record the effect of D-3-HB on both kinases. The citrate level is increased in hearts perfused with D-3-HB, and it is known that citrate inhibits AMPK phosphorylation in rat hypothalamus [58], suggesting a possible mechanism whereby D-3-HB inhibits AMPK. Moreover, acetoacetate increases the phosphorylation of extracellular signal-regulated kinase ½ (ERK1/2) and p38 MAPK in hepatocytes [59] and we found that DNP stimulates acetoacetate utilization in the heart from normal and chronic diabetic animals [60]. It is conceivable that D-3-HB protects the cells against ATP depletion by providing NADH to the electron transport chain. D-3-HB may either limit or counteract the effect of DNP (decrease in mitochondrial membrane potential and ATP/ADP ratio) by providing a high supply of reducing equivalents to the respiratory chain, since it is oxidized directly in the mitochondrial matrix. D-3-HB further compensates for the energy deficit caused by either DNP or metabolic stress. DNP contributes to proton leakage, which keeps the cell NAD^+/NADH ratio sufficiently high and allows the required carbon metabolism to continue. It has been proposed that high NAD^+/NADH ratio enhances AMPK activity [61], but other findings lack any sufficient support [62].

Fasting (24 or 48h) is associated with the stimulation of cardiac D-3-HB utilization [63]. AMPK is activated by fasting and is suppressed by Ara-A [64]. Also, Ara-A decreased AMPK phosphorylation in the heart in response to the deprivation of glucose [65]. Taken together, these findings showed that the modulation of either the AMPK or p38 MAPK signaling pathways had a role in regulating D-3-HB utilization. Our study also showed that DNP stimulated D-3-HB utilization in the isolated perfused heart is mediated by the AMPK signaling pathway, and Ca^{2+} has a significant role in regulating D-3-HB utilization through its activation of CaMKK, which then activates AMPK. Both mechanisms, altering either the ATP/AMP or Ca^{2+} levels, could independently contribute to the enhancement of D-3-HB utilization.

We proceeded to observe that mimicking chemical hypoxia with DNP, or chemical ischemia-reperfusion with anisomycin, or activating AMPK/p38 MAPK stimulated the utilization of D-3-HB and the inhibition of either kinases are associated with the reduction of D-3-HB utilization in the isolated perfused heart. AMPK and p38 MAPK are stimulated in response to ischemia [8,16,33]. No-flow ischemia stimulates AMPK α_2 and α_1 activities and is most likely mediated by LKB1 (AMPKK) and CaMKK, respectively [49] and AMPK α_2 has a greater dependence on AMP [66]. Cardiac AMPK activity is stimulated by ischemia and reperfusion [9]. Since it is well known that such stressor effects are mediated by AMPK/p38 MAPK, our data supports the idea that both kinases are involved in the regulation of D-3-HB metabolism in the heart. Since PD-169316 and STO-609 abolished D-3-HB utilization stimulated by ischemia-reperfusion, our data also support that both AMPK and p38 MAPK mediate the ischemia-reperfusion effect. We encountered a few limitations that include having not measured either the AMPK or p38 MAPK activities and using the available selective activators and inhibitors of the both kinases.

Conclusion

We conclude with the following key findings: DNP (an activator of both cardiac AMPK and p38 MAPK), anisomycin (a selective activator of p38 MAPK), and ionomycin (a CaMKK activator) stimulate D-3-HB utilization in heart, and this stimulation is either partially or completely abolished by selective inhibitors of the above mentioned kinases. The chemical stress is mediated independently by changes in Ca^{2+} concentration and /or AMP/ATP levels, through the AMPK/p38 MAPK signaling pathways. Global no-flow ischemia stimulates D-

3-HB utilization through the same signaling pathways. D-3-HB regulates glucose utilization in the heart via AMPK/p38 MAPK signaling cascades.

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Conflict of Interest

Nothing to declare.

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