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RESEARCH ARTICLE

THE EFFECT OF DIABETES ON ACETOACETATE METABOLISM IN HEART.

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Abstract

Background Aims: In this study, the effects of perfusion pressure, insulin, L-carnitine, propionate and 2,4-dinitrophenol on the utilization and oxidation of acetoacetate were investigated in the isolated non-working perfused heart from normal and diabetic rats.

Materials and Method: Hearts from Male Wistar albino rats were used. In the diabetic subgroup, Diabetes was induced by an intravenous injection of alloxan. The hearts were perfused at a perfusion pressure of 40 or 80 mmHg for 1 h with Krebs-Henseleit Medium, with the concentrations of calcium and magnesium halved, and oxygenated by equilibration with 5% carbon dioxide and 95% oxygen. Determination of acetoacetate and D-3-hydroxybutyrate levels were made by the method of Mellanby and Williamson and Williamson and Mellanby respectively, and comparison between groups was done using the two-tailed Student's t-test for independent samples.

Results

- Increasing Perfusion Pressure: No effect in both normal or diabetic hearts.
- Insulin: No effect on acetoacetate utilization.
- Diabetes: A decrease in the utilization and oxidation of acetoacetate and the production of D-3-hydroxybutyrate.
- L- Carnitine:
  - Normal hearts: No effect on acetoacetate utilization.
  - Diabetic hearts: Acetoacetate utilization was enhanced
- Propionate:
  - Normal hearts: Inhibited utilization of acetoacetate without affecting the rate of oxidation. The rate of D-3-hydroxybutyrate production was inhibited.
  - Diabetic hearts: Enhanced the utilization and oxidation of acetoacetate while reducing the production of D-3-hydroxybutyrate
- Dinitrophenol:
  - Normal hearts: Enhanced the utilization and oxidation of acetoacetate and decreased the production of D-3-

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hydroxybutyrate
- Diabetic hearts: Utilization and oxidation of acetoacetate were enhanced without effect on production of D-3-hydroxybutyrate.

Conclusion: Diabetes inhibits and insulin has no significant effect on myocardial acetoacetate utilization and oxidation. Insulin, L-carnitine, or propionate are not suitable to ameliorate the utilization of acetoacetate in hearts from normal or diabetic animals, whereas Dinitrophenol enhances the catabolism of acetoacetate.

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Introduction:-

It has long been known that the isolated non-working perfused heart utilizes acetoacetate as a preferred substrate of oxidation, even in the presence of glucose and insulin[1, 2], whereas acetoacetate and/or D-3-hydroxybutyrate (D-3-HB) as sole substrate(s) do not maintain cardiac function in the isolated working heart[3]. Ketone bodies oxidation is preserved in advanced heart failure to maintain adequate myocardial function[4]. Insulin inhibits the utilization and oxidation of acetoacetate in hearts from normal rats[1, 3], and in contrast, insulin stimulates the utilization and oxidation of D-3-HB in hearts from normal and diabetic rats[5, 6]. Propionate has been shown to enhance the utilization and oxidation of acetoacetate in the non-working heart[7] and improves heart contractile function in the working heart[8], whereas propionate inhibits the utilization, but not the oxidation of D-3- HB[9]. Carnitine enhances the removal of acetoacetate in vivo and in vitro studies[10, 11, 12, 13], but has no effect on the removal of D-3-HB[12, 13]. Ischemia-reperfusion stimulates myocardial D-3-HB utilization[14]. The deletion of succinyl-CoA:3-oxoacid-CoA transferase (SCOT) in mouse hearts promotes accelerated pathological remodeling[15] (the rate-limiting enzyme for myocardial oxidation of β-hydroxybutyrate and acetoacetate) and in advanced human heart failure the myocardial utilization of D-3-HB is increased and there is a significant increase in the expression of the gene encoding succinyl-CoA:3-oxoacid-CoA transferase[16]. In mouse models, cardiac hypertrophy and failure causes increased expression of β-hydroxybutyrate dehydrogenase 1 (BDH1) and the heart is shifted to ketone bodies as a significant source of energy[17]. The data support the importance of ketone bodies as a source of energy and their significant regulatory role in heart metabolism[18]. The utilization and oxidation of D-3-HB were inhibited in hearts from diabetic rats[5, 6]. No data were found in the literature on the effect of diabetes on the metabolism of acetoacetate in heart, and very limited data is available on acetoacetate as a sole substrate in the heart[19]. Although the first step of D-3-HB oxidation is its conversion to acetoacetate there is a difference in the reported effects of insulin, propionate, and L-carnitine on the metabolism of acetoacetate and D-3-HB, this encouraged us to carry out this investigation. An attempt was also made to improve the utilization and oxidation of acetoacetate in the heart.

Materials and Methods:-

Animals:-

Male Wistar albino rats weighing 200-350 g were housed at a constant temperature of 22°C ± and a 12 h light/ 12 h dark cycle. The animals were given continuous access to tap water and a rat pellet diet containing 13% protein and 3% fat[20].

Induction of Diabetes:-

The rats were made diabetic by a single intravenous injection of alloxan (40 mg/kg). Rats were used 4 weeks after alloxan administration. There was about 30% reduction the body weight of diabetic rats. The concentration of blood glucose was about 16.6 mM[5].

Chemicals:-

All chemicals, of the highest available grade were obtained from Sigma London Chemical Corp. Ltd., U. K.

Analytical Methods:-

The determination of acetoacetate and D-3-HB were made by the method of Mellanby and Williamson[21] and Williamson and Mellanby[22] respectively.

Media:-
Hearts from normal or diabetic rats were perfused at a perfusion pressure of 40 mmHg or 80 mmHg for 1 h with Krebs-Henseleit Medium[23], modified in that the concentration of calcium and magnesium were halved (MKHM), and oxygenated by equilibration with 5% carbon dioxide and 95% oxygen. The initial concentrations of acetoacetate (Li-salt), propionate, L-carnitine, DNP, and insulin as shown in the results.

Perfusion Method:-
Hearts were perfused as described previously[20]. Hearts were perfused at a perfusion pressure of 40 mmHg or 80 mmHg; the perfusate temperature was 37°C. The technique involves the continuous infusion of fresh perfusate into a volume of recirculating perfusate that is kept constant by balanced withdrawal. Perfusate was introduced at 32.6± 0.1 (80) $ml.h^{-1}$. Fractions were collected for 5 minutes, alternate functions were analysed to determine acetoacetate and D-3-HB concentrations. The mechanical performance (rate, vigour and rhythm) of the heart were noted periodically[24]. These parameters along with the metabolic stability were used as criteria of a successful preparation. Coronary flow was measured at the end of each experiment, 48±1 (6) and 109±7(9) ml.g.dry $wt^{-1}.min^{-1}$. at 40 and 80 mmHg respectively.

Calculation and statistical analysis:-
Calculation as described before[20]. Comparisons between groups were assessed using the two-tailed Student's t-test for independent samples.

Results:-

Effect of Perfusion Pressure:-
Increasing the perfusion pressure from 40 mmHg to 80 mmHg in hearts from normal (Groups 1 and 2) or diabetic animals (Groups 7 and 8), had no significant effect on the utilization and oxidation of acetoacetate and D-3-HB production.

Effect of Diabetes:-
At a perfusion pressure of 80 mmHg, the rates of acetoacetate utilization and oxidation and D-3-HB production were inhibited by 38%, 30% and 36% ($P<0.003$, $P< 5\text{-}8\ e^{-5}$, $P< 0.001$) respectively, in hearts from diabetic animals (Group 8), whereas at 40 mmHg the rates of acetoacetate utilization and oxidation, and D-3-HB production were inhibited by 42%, 46%, 36% ($P< 2.26e^{-4}$, $P< 0.003$, $P<4.05e^{-4}$) respectively (Group 7).

Effect of Insulin:-
Insulin had no significant effect on the utilization of acetoacetate in hearts from normal or diabetic animals (Groups 3 and 9).

Effect of L-Carnitine:-
L-Carnitine had no effect on acetoacetate utilization in hearts from normal animals (Group 4), whereas in hearts from diabetic animals (Group 10) the rate of acetoacetate utilization is enhanced by 16% ($P< 0.02$) without effecting the rate of acetoacetate oxidation, but the production of D-3-HB was increased by 44% ($P< 0.05$).

Effect of Propionate:-
In hearts from normal animals (Group 5) propionate inhibited the utilization of acetoacetate by 20% ($P< 0.02$) without affecting the rate of oxidation, but the rate of D-3-HB production is inhibited by 72% ($P< 6.1e^{-8}$). On the other hand, in hearts from diabetic animals (Group 11) propionate enhanced the utilization and oxidation of acetoacetate by 46% and 78% ($P< 0.003$, $P<0.002$) respectively and the production of D-3-HB was reduced by 38% ($P< 0.1$).

Effect of 2,4-Dinitrophenol:-
Dinitrophenol enhanced the utilization and oxidation of acetoacetate by 35% and 56% ($P< 7.27e^{-5}$, $P<7.82e^{-6}$) respectively and decreased the production of D-3-HB by 18% ($P< 0.003$) in hearts from normal rats, whereas in hearts from diabetic rats utilization and oxidation of acetoacetate were enhanced by 60% and 80% ($P< 0.003$, $P< 0.004$) respectively, without affecting the production of D-3-HB.

Discussion:-

Although the rate of coronary flow was increased (from 48 to 109 ml.g.dry wt^{-1} $min.^{-1}$) on increasing the perfusion pressure from 40 to 80 mmHg, the rates of acetoacetate utilization and oxidation were not affected nor was the rate of D-3-HB production. On the other hand, increasing the perfusion pressure 3-fold (25 to 75 mmHg) increases the oxidation of acetoacetate about 34% without affecting the acetoacetate utilization, but decreases the production of D-3-HB by 45%[7]. Comparing the rates of acetoacetate oxidation of non-working rat heart[1] and working heart[3] indicates that there is no effect of work-load on the oxidation of acetoacetate. Whereas, the utilization and oxidation of D-3-HB was enhanced by 30% and 44% respectively[9] on doubling the perfusion pressure (40 to 80 mmHg).

Chronic diabetes (4-weeks) inhibited the utilization and oxidation of acetoacetate and D-3-HB production, this finding is compatible with the decrease in the activity of 3- hydroxybutyrate dehydrogenase [EC 1.1.1.30] (51%) in heart mitochondria[25] . Since the oxidation of acetoacetate is inhibited, it is most likely that the activity of 3-oxoacid CoA-transferase [EC 2.8.3.5] is decreased. Grinblat et al.[25] reported 50% decrease in heart mitochondria three months after onset of diabetes, whereas the acetoacetyl CoA - thiolase [EC 2.3.1.9] was not affected. Sultan[6] reported that diabetes (4 weeks) inhibits the utilization and oxidation of D-3-HB. Therefore, the rise of the blood level of ketone bodies during diabetes is due to over production and the decrease in the utilization and oxidation of ketone bodies.

Since insulin has no effect on acetoacetate utilization and oxidation, and enhances the utilization and oxidation of D-3-HB[5,6], introducing insulin therapy in ketosis, if it does not improve the overall utilization of ketone bodies, will not deteriorate the case. The reduction in the rate of acetoacetate utilization and oxidation in diabetes is unlikely due to the lack of insulin. It seems that the defect in the catabolism of acetoacetate in diabetes can be corrected by introducing anaplerotic substance such as propionate which could enrich citric acid cycle with succinyl CoA (an essential metabolite for acetoacetate oxidation), but the inhibitory effect of propionate on D-3-HB utilization[9] preclude the use of propionate to ameliorate ketone bodies catabolism. It has been found that propionate caused a drastic reduction of free CoA and L-carnitine in the heart, Di Lisa et al.[26].

L-Carnitine had no effect on acetoacetate utilization in heart from normal rats, whereas, the 16 % increase of acetoacetate utilization in diabetic state is compensated by the increase in the rate of D-3-HB production, since the rate limiting step of acetoacetate and D-3-HB oxidation, 3-oxoacid CoA-transferase [EC 2.8.3.5], is inhibited in diabetes. Although, it has been reported that carnitine enhances the removal of acetoacetate in diabetes[12, 13], L-carnitine is not suitable to ameliorate the oxidation of acetoacetate in normal or diabetic states, moreover L-carnitine has no effect on D-3-HB utilization or oxidation in the perfused rat heart (unpublished observation), and does not stimulate D-3-HB utilization in heart mitochondria[27]. L-Carnitine is reported to increase the concentration of tissue CoA[11], according to Russell and Taegtmyere[8] such a change could improve the oxidation of acetoacetate, but the presented data did not support that CoA alone could be a limiting factor for acetoacetate oxidation. Propionate and L-carnitine addition alone to acetoacetate had negligible effects on contractile function, Russell et al.[28].

Dinitrophenol (DNP) is an un-coupler (uncouples oxidative phosphorylation), its stimulatory effect on the utilization and oxidation of acetoacetate is likely mediated through its capability to alter the $NADH/NAD^+$ ratio in the mitochondria. The decrease in D-3-HB production in the normal state and the increase in the percent oxidation of acetoacetate in the diabetic state support such a suggestion, moreover, DNP enhances the utilization and oxidation of D-3-HB in isolated perfused heart in a concentration-dependent manner[14], by decreasing myocyte $NADH^{+}$[29], raising Ca^{+2} cytosolic concentration, and AMP/ATP ratio[30, 31] through stimulating AMPK/p38 MAPK [32].
The regeneration of NAD^+ could enhance a-ketoglutarate dehydrogenase (a-KGDH) which is reported to be inhibited by acetoacetate[10], and thereby the oxidation of ketone bodies is enhanced by providing succinyl CoA.

In conclusion, increasing perfusion pressure, and giving insulin, failed to improve the catabolism of acetoacetate in hearts from normal or diabetic animals. Acetoacetate utilization and oxidation are inhibited in hearts of diabetic animals. L-Carnitine and propionate are not good candidates to ameliorate the catabolism ofacetoacetate. 2,4-Dinitrophenol enhanced the utilization and oxidation of acetoacetate in hearts from normal or diabetic animals suggesting that $NADH/NAD^+$ ratio plays an important role in the regulation of ketone bodies' metabolism. It is possible that dinitrophenol stimulated acetoacetate oxidation act through AMPK/ p38 MAPK signalingpathway as suggested for D-3-HB[14].

Table 1:-

Hearts from normal (Groups 1 to 6) and diabetic (Groups 7 to 12) rats were perfused with MKHM containing acetoacetate (Ac) as indicated in the table for 60 minutes. The addition of other substances are shown in the table, and their initial concentrations are insulin (2mU.m1-1), L-carnitine (5 mM), propionate (4 mM), DNP (0.05 mM). The perfusate concentration of Ac and D-3-HB and the rates of utilization, oxidation and production were given for the last 30 minutes of perfusion period. The perfusion pressure is 80 mmHg except for groups 1 and 7, it is 40 mmHg and indicated by *. Values are means ± SEM for the numbers of observations in parentheses.

Group	Addition	Initial conc. Ac (mM)	Perfusate con. Ac (mM)	Perfusate con. D-3-HB(mM)	Utilization of Ac	Oxidation of Ac	Production of D-3-HB
1*	----	5.39 ± 0.07(6)	3.33±0.13	0.69±0.03	398±16	263±18	135±5
2	----	5.25±0.09(9)	3.19±0.08	0.56±0.02	406±18	295±16	111±4
3	Insulin	5.26±0.06(9)	3.25±0.11	0.54±0.03	416±12	318±21	111±6
4	L-Carnitine	5.28±0.07 (6)	3.31±0.08	0.46±0.07	416±22	320±28	96±12
5	Propionate	5.04±0.1(6)	3.48±0.3	0.15±0.04	319±27	288±22	31±7
6	DNP	5.30±0.06(6)	2.68±0.16	0.43±0.03	551±15	460±14	91±4
7*	----	5.38±0.03(6)	4.33±0.04	0.40±0.01	230±11	143±9	87±3
8	----	5.20±0.03(6)	4.16±0.1	0.20±0.05	251±18	181±23	71±13
9	Insulin	5.08±0.06(9)	3.84±0.1	0.30±0.03	272±17	205+20	67±8
10	L-Carnitine	5.32±0.05(6)	4.02±0.2	0.45±0.04	290±23	188±21	102±5
11	Propionate	5.19+0.08(6)	3.68±0.1	0.19±0.04	366±23	322±25	44±7
12	DNP	5.14±0.08(5)	3.44±0.16	0.33±0.03	401±34	325±31	76±4

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