Hoppe-Seyler's Z. Physiol. Chem.
Bd. 356, S. 839 - 844, Juni 1975

Effects of Arginine Homologues and other Guanidino Compounds on the ATP Level and Glucose Oxidation in Isolated Fat Cells

Friedemann Schwegler and Wieland Stock

(Received 27 January 1975)

Dedicated to Prof. Dr. Dr. G. Weitzel on his 60th birthday

Summary: The arginine homologues 2-amino-3-guanidinopropionic acid, 2-amino-4-guanidinobutyric acid and 2-amino-6-guanidinocaproic acid (= homoarginine) were synthesized and transformed into their methyl esters. The latter, together with arginine methyl ester, arginine diethylamide and some guanidino compounds without the arginyl structure (agmatine, isopentylguanidine and n-butylbiguanide) were examined with regard to their behaviour on isolated fat cells, concerning the adrenalin-induced depression of the ATP level and the stimulation of glucose oxidation.

The homoarginyl and arginyl derivatives counteracted the effect of adrenalin by re-elevating the ATP level, and thus they exerted an insulin-like activity. The esters were slightly active, whereas the arginine diethylamide and agmatine had a marked effect. The shorter homologues of arginine were totally inactive. However isopentylguanidine and butylbiguanide followed the effect of adrenalin: they additionally lowered the ATP level and therefore they acted in opposition to insulin.

For comparative reasons the same compounds were tested with regard to their effects on glucose oxidation. The results were consistent with those quoted above: the homoarginyl and arginyl derivatives (agmatine included) forced the glucose oxidation similarly to insulin, the shorter homologues were inactive, isopentylguanidine and butylbiguanide decreased it.

Wirkung von Argininhomologen und anderen Guanidinoverbindungen auf ATP-Spiegel und Glucose-Oxidation in isolierten Fettzellen

Zusammenfassung: Die Argininhomologen 2-Amino-3-guanidinopropionsäure, 2-Amino-4-guanidinobuttersäure und 2-Amino-6-guanidinocapronsäure (= Homoarginin) wurden synthetisiert und in ihre Methylester überführt. Diese wurden zusammen mit Argininmethylester, Arginindiäthylamid und einigen Guanidinoverbindungen ohne Arginylstruktur (Agmatin, Isopentylguanidin, n-Butylbiguanid) in isolierten Fettzellen auf ihr Verhalten im System der adrenalininduzierten Senkung des ATP-Spiegels sowie der Stimulierung der Glucoseoxidation untersucht.

Die Homoarginyl- und Arginylderivate wirkten dem Adrenalineffekt entgegen, d.h. sie erhöhten

---

Address: Dr. Friedemann Schwegler, Physiologisch-Chemisches Institut der Universität Tübingen D-74 Tübingen, Hoppe-Seyler-Str. 1.

den ATP-Spiegel und wirkten somit insulinähnlich. Dabei waren die Ester schwach, Argininodiäthylamid und Agmatin stark wirksam. Die kürzeren Homologen des Arginins waren inaktiv, Isopentylguanidin und n-Biguanid dagegen folgten der Adrenalinwirkung: sie senkten den ATP-Spiegel noch weiter und verhielten sich damit entgegengesetzt zur Insulinwirkung.

Zum Vergleich wurden dieselben Verbindungen im System der Glucoseoxidation in isolierten Fettzellen untersucht. Man erhielt parallele Ergebnisse: Die Homoarginyl- und Arginylderivate (einschl. Agmatin) steigerten wie Insulin die Glucoseoxidation, die kürzeren Argininhomologen waren inaktiv, Isopentylguanidin und n-Butylbiguanid setzten die Glucoseoxidation herab.

In preceding studies, Weitzel et al.[1-3] demonstrated that certain guanidino compounds, primarily *derivatives of arginine*, and also agmatine as the decarboxylation product of arginine, have an *insulin-like* activity in several insulin-sensitive systems. On the one hand they increase the glucose uptake and glycogen content of rat diaphragm in vitro, on the other they stimulate the glucose oxidation in adipose tissue cells. In addition they are able to antagonize effects which are induced in the latter-mentioned system by lipolytic substances like adrenalin.

Some other guanidino compounds, which were studied under the same conditions, react similarly to insulin, others have the opposite effect. A comparable behaviour was demonstrated for some polyamines by Lockwood and coworkers[4]. Recently Bihler and Jeanrenaud[5] pointed out a further detail of the antilipolytic effect of insulin. In fat cells insulin increases the *ATP level*, which has previously been decreased by adrenalin action. This system, not used hitherto in the investigations of Weitzel et al., should indicate if there are the same specific differences between two groups of guanidino compounds as demonstrated in the above-mentioned experiments: one group derived from arginine, the other without an arginyl structure. With regard to results obtained by other methods with this procedure the specificity of the structure of the two groups should be defined.

Moreover some substances, not used till now, were examined: both 2-amino-3-guanidinopropionic-, 2-amino-4-guanidinobutyric-, and 2-amino-6-guanidinocaproic acids (= homoarginine) were synthesized and transformed into derivatives suitable for the biological test. Thereby, the measurements were based on the complete set of α-amino-ω-guanidino carbonic acids from C_4 up to C_7. To be able to compare the measurements of all compounds it was also necessary to test them in a well-known system. For this purpose we monitored the glucose oxidation in isolated fat cells of rat adipose tissue.

Methods

I. Syntheses

All compounds with a free guanidino group were tested by thin-layer chromatography with regard to their uniformity (CE plates from Riedel de Haen, solvent system: phenol/water (75:25), development with Sakaguchi reagent).

1) L-2-Amino-3-guanidinopropionic acid (see also l.c.[6])

a) p-Tosyl-L-asparagine (I)

50 g *p*-tosyl chloride dissolved in 200 m*l* acetone and 35.2 g L-asparagine · 1 H_2O in 210 m*l* 1N NaOH were mixed by stirring. Within the next 40 min an equal quantity of 210 m*l* 1N NaOH was added in small portions at room temperature. Subsequently the remaining tosyl chloride was separated by filtration. The acetone was removed from the solution under reduced pressure and the pH adjusted to 3. After several hours in the refrigerator crystals separated. Recrystallization from ethanol/water (70:30) gave a product with m.p. 188 - 190 °C. Yield: 55.7 g (88.3%).

$C_{11}H_{14}N_2O_5S$ (286.0)

Calc. C 46.15 H 4.93 N 9.79
Found C 46.05 H 4.94 N 9.81

$[\alpha]_D^{20}$: − 10.3° (c = 1.5 in dimethylformamide)

b) N-2-Tosyl-L-2,3-diaminopropionic acid (II)

12 m*l* bromine was dropped into a stirred solution of 3N ice-cold NaOH. 55 g of compound I was dissolved in 155 ml 3N NaOH and added to the solution while cooling. After heating the mixture to 80 °C for about 15 min the pH was adjusted to 7 with 5N HCl. After 12 h in the refrigerator the crystalline product was collected, washed with 50 m*l* ice-cold water and recrystallized from 20% aqueous acetic acid. The product was dried over KOH at 120 °C and 12mm. m.p. 210 - 220 °C (decomp.). Yield: 33.6 g (68%).

$C_{10}H_{14}N_2O_4S$ (258.3)

Calc. C 46.49 H 5.46 N 10.85
Found C 46.57 H 5.57 N 10.73

c) L-2-Tosylamino-3-guanidinopropionic acid (III)

30 g II was dissolved in 140 ml 4N NaOH. To this solution 102 g *O*-methylisourea sulphate[7] was added and kept for 3 days at room temperature. The resulting precipitate was filtered, dissolved in a small amount of water and the pH adjusted to 6 with glacial acetic acid. The precipitate was separated and recrystallized from hot water which was acidified with acetic acid. The product was dried over P_2O_5 for 12 h at 40 °C and 12 mm and had a m.p. 190 - 195 °C. Yield: 27.7 g (73%).

$C_{11}H_{16}N_4O_4S \cdot 2\,H_2O$ (336.4)

Calc. C 39.27 H 5.95 N 16.66
Found C 39.41 H 5.92 N 16.47

d) L-2-Amino-3-guanidinopropionic acid

27.7 g III and 22 g phenol were added to 300 ml of a 35% solution of HBr in glacial acetic acid. The solution was then kept at 40 °C for 36 h. The mixture was diluted and the precipitate which resulted was washed several times with ether. The product was dissolved in a small amount of water and filtered through a column of Amberlite IRA 400 $OH^{\ominus}$ (6.5 x 28 cm). The column was eluted with water. The ninhydrin-positive fractions were collected and the pH adjusted to 7.0 with HCl. After evaporation the product was precipitated from water with ethanol. m.p. 210 °C. Yield: 10.4 g (96%).

$C_4H_{10}N_4O_2 \cdot HCl$ (182.7)

Calc. C 26.31 H 6.07 N 30.68
Found C 26.11 H 6.05 N 30.40

$[\alpha]_D^{22}$: ± 14.4° (c = 2.8 in H_2O)

2) L-2-Amino-4-guanidinobutyric acid

To 38.2 g L-2,4-diaminobutyric acid · 2 HCl[8] dissolved in 150 ml H_2O an excess of basic copper carbonate was added and the mixture was boiled for about 45 min. When the CO_2 evolution had finished, the remaining excess of copper carbonate was filtered off and, after addition of 52.8 g *O*-methylisourea sulphate, the pH of the solution was adjusted to 10.5 with 5N NaOH. The solution was kept at room temperature for 10 days. After acidifying with HCl the copper was precipitated with H_2S. After separation from CuS the filtrate was applied to an anionic exchange column (IRA 400, $OH^{\ominus}$, 5 x 60 cm) and eluted with water. The ninhydrin-positive fractions were collected and lyophylized. This material was taken up in a small amount of water, acidified with HCl to about pH 1 and treated with an excess of aniline. From this mixture the product was precipitated with ethanol. Recrystallization twice from aqueous ethanol gave the guanidino compound unchanged by further recrystallization with m.p. 108 - 109 °C. Yield: 21.2 g (51%).

$C_5H_{12}N_4O_2 \cdot HCl \cdot H_2O$

Calc. C 28.26 H 6.98 N 26.16
Found C 28.27 H 7.02 N 26.36

$[\alpha]_D^{23}$: + 29.6° (c = 1.1 in 5N HCl)

3) L-2-Amino-6-guanidinocarbonic acid (= homoarginine)

The synthesis was performed according to l.c.[9] by reaction of the Cu complex of L-lysine with *O*-methylisourea sulphate.

4) Methyl ester of the arginine homologues

The esterification was achieved by the thionyl chloride method according to l.c.[10].

a) L-2-Amino-3-guanidinopropionic acid methyl ester

m.p. 172 °C.

$C_5H_{12}N_4O_2 \cdot 2\,HCl$ (233.0)

Calc. C 25.75 H 6.00 N 24.03
Found C 25.99 H 6.04 N 23.96

b) L-2-Amino-4-guanidinobutyric acid methyl ester

m.p. 162 °C.

$C_6H_{14}N_4O_2 \cdot 2\,HCl$

Calc. C 29.13 H 5.66 N 22.65
Found C 29.05 H 5.59 N 22.37

c) L-homoarginine methyl ester

m.p. 120 - 125 °C.

$C_8H_{18}N_4O_2 \cdot 2\,HCl$ (275.2)

Calc. N 20.35
Found N 20.27

$[\alpha]_D^{24}$: + 14.2° (c = 2 in H_2O)

Isopentylguanidine[1], L-arginine diethylamide[3] and L-arginine methyl ester[1] were a gift from Prof. Guglielmi, agmatine (4-aminobutylguanidine) and n-butylbiguanide were purchasable products. All other reagents were products of Merck A.G. Darmstadt, Germany, collagenase was from Worthington (Freehold, N.Y., USA).

II) Biological methods

1) Measurement of the ATP level in fat cells

Fat cells were obtained from male Wistar rats, weighing 100 - 120 g, (*Mus rattus*, Höhenkirchen, Germany), which were fed with Altromin ad libitum.

The isolation of fat cells was originally described by Rodbell[11], some modifications are shown in l.c.[1]. The incubation of the cells followed the procedure in l.c.[3]. The suspension contained 5 - 8 · 10^5 cells per ml. The concentration of adrenalin was 1 µg per ml incuba-

tion medium throughout. After incubation for 90 min the cells were treated as described in l.c.[4]. ATP was measured according to the method of Kalbhen and Koch[12]. The luciferin-luciferase system was prepared daily from 50 mg of dried firefly abdomina (Sigma London). After grinding with sea-sand the mixture was extracted with a 0.025M glycylglycine buffer (0.02M $MgSO_4$) pH 7.4 instead of the arsenate buffer as used by the above mentioned authors. The extraction was following by centrifugation at 3 000 × *g*. The sediment was washed twice and the combined filtrate and washings were diluted to 250 m*l* with buffer.

Within the first two hours a certain decrease of the enzyme activity took place, whereas the activity remained approximately constant during the next two hours. The measurements were done during this latter period.

10 Seconds after mixing 500 μl of the sample with 5 m*l* of enzyme extract the luminescence was measured in a liquid scintillation counter (Packard mod. 3003), set for counting ^3H, over a period of 0.1 min. The linear function between the root of counts per 0.1 min and the ATP concentration as shown in l.c.[4] was found to apply.

2) Glucose oxidation in fat cells

Insulin and substances with insulin-like activity stimulate the oxidation of glucose in isolated cells of the rat adipose tissue. This is measured by the determination of $^{14}CO_2$ formed from [1-^{14}C]glucose by the cells. These experiments were carried out as described in l.c.[1].

Results

1) Syntheses

Kurtz[13] was the first to describe the possibility of synthesizing arginine by the reaction of derivatives of isourea with ornithine, which must be protected at its α-aminogroup with copper. With some variations this method was employed to synthesize homologues of arginine. In the case of the homologues prolonged or shortened by one methylene group, this method was successful. However, according to the reference in l.c.[13], it was impossible to synthesize in this manner the homologue shortened by two methylene groups. The functional groups seemed to be too close to each other to allow the desired reaction. The latter compound was synthesized according to Rudinger's method[6]. As a reliable and easily obtainable guanylation reagent, *O*-methylisorea sulphate[7] was used. The esters of the shorter homologues became easily crystalline, the one of the longer homologue crystallized after long standing. As far as we know, the esters of the arginine homologues are not described in the literature.

2) Biological measurements

a) ATP level in fat cells

The results of the ATP measurements are shown in Tab. 1. The % values refer to the ATP level of the controls, which was fixed as 100%. The absolute values of the non-induced cells reached 100 ± 18 nmol ATP/g dry weight of fat cells. This level was slightly higher than that described in l.c.[4]. The addition of adrenalin lowered the ATP level to about one fifth of that in the controls throughout all assays (19 ± 2 nmol ATP/g). Values are given in column 2 in Tab. 1.

To elucidate the efficiency of the particular substances, their effects are compared to that of insulin. 4.2 ng insulin per m*l* medium raised the adrenalin induced decrease of the ATP level to 90.7% of the initial content (36 assays). The third column shows the values for the different substances. The concentrations were always 1 μM per m*l*. The results of the guanidino compounds without the structure of arginine, these being isopentylguanidine and n-butylbiguanide, are remarkable. They enhance the effect of adrenalin in a very significant manner. Therefore one may consider them as lipolytic agents.

The compounds with an arginyl structure, however, had to be divided into two groups. 1) The derivatives of the shorter homologues of arginine. As can be seen in Tab. 1 they show no effect; that is neither an increase nor a decrease of the adrenalin effect. 2) The derivatives of arginine and the ester of the longer homologue lowered the effect of adrenalin. The values of the methyl esters of arginine and homoarginine were slight, but still significant. The powerful effect of one derivative of arginine, argininediethylamide, is striking. It considerably suppressed the activity of adrenalin and is the derivative with the strongest activity of all compounds listed in Tab. 1. In addition agmatine likewise markedly suppressed the effect of adrenalin and showed thereby insulin-like activity.

Table 1. Influence of guanidino compounds on the adrenalin-induced decrease of the ATP level in fat cells.
ATP level of the untreated cells = 100%. For details concerning the absolute values and the experimental conditions see the sections "methods" and "results". *n* = number of experiments.

Substance	ATP concentration in % of the control				
	1 µg Adrenalin/m*l*	1 µg Adren. + 1 µM subst./m*l*	Deviation *s*	$p <$	*n*
Isopentylguanidine	19.6	8.4	± 0.6	0.01	11
n-Butylbiguanide	17.9	6.7	± 0.5	0.01	10
2-Amino-3-guanidinopropionic acid methyl ester	18.3	18.5	± 1.8	0.25	11
2-Amino-3-guanidinobutyric acid methyl ester	18.3	18.1	± 1.7	0.25	11
Arginine methyl ester	19.6	21.7	± 3.0	0.01	12
Homoarginine methyl ester	17.9	19.7	± 3.3	0.05	10
Argininediethylamide	20.7	49.7	± 3.2	0.001	10
Agmatine	20.7	26.6	± 2.4	0.005	11

Table 2. Influence of guanidino compounds on the glucose oxidation in fat cells.
For details concerning absolute values and the experimental conditions see section "methods" and "results". The values marked by indices are adopted from the respective publications. *n* = number of experiments.

Substance	Formation of $^{14}CO_2$ from [1-^{14}C]glucose in % of the control (control = 100%)			
	Mean	Deviation *s*	$p <$	*n*
Isopentylguanidine[1]	69	± 3.0	0.001	12
n-Butylbiguanide[1]	57	± 4.5	0.001	10
2-Amino-3-guanidinopropionic acid methyl ester	101	± 17.3	0.3	10
2-Amino-4-guanidinobutyric acid methyl ester	98	± 18.6	0.25	10
Arginine methyl ester[1]	118	± 7.5	–	16
Homoarginine methyl ester	178	± 19.0	0.001	10
Argininediethylamide[3]	181	± 14.7	0.001	28
Agmatine[1]	171	± 13.0	0.001	25

b) Glucose oxidation in fat cells

Again the glucose oxidation figures of the controls were fixed as 100%. This corresponded to a gain of 1520 cpm per 5 x 10^5 cells per m*l*. The values varied from day to day between 800 and 2000 cpm. Within the same day the deviations were low (±4%) and therefore only these results were compared. On the average, the stimulation rate induced by 4.2 ng insulin per m*l* was 800%.

As demonstrated in Tab. 2, homoarginine methyl ester showed effects similar to those established previously for arginyl compounds by our research group[1]. These results are also listed for comparison in this Table. All these substances stimulated the glucose oxidation. The shortened homologues (L-2-amino-3-guanidinopropionic acid- and L-2-amino-4-guanidinobutyric acid methyl ester) however showed neither a positive nor a negative effect.

In contrast to the compounds with an arginyl structure isopentylguanidine and butylbiguanide showed an effect opposite to that of insulin, as in the ATP measurements. These findings have already been observed by Ditschuneit and co-workers[14] at substantially lower concentrations during the examination of butylbiguanide.

Discussion

This work was done as a supplement to the results shown by Weitzel et al.[1-3]. As a new insulin-sensitive system, the level of ATP in isolated fat cells was used. Additionally some shorter and longer homologues of arginine were examined for the first time.

In the case of the arginyl compounds already investigated (incl. agmatine) we could corroborate the results described before. These findings are broadened to the extent, that the homoarginine methyl ester followed the effects of the derivatives of arginine. Their behaviour in the glucose oxidation was the same as in the ATP system. They all followed qualitatively the effects of insulin. In both systems the results of the shorter arginine homologues were in agreement. In our investigations they surprisingly proved to be biologically inactive. Isopentylguanidine and n-butylbiguanide, however, behave in both systems oppositely to the arginyl- and homoarginyl compounds. Although they have the characteristic guanidino-group too, this fact does not seem to be sufficient to produce similar biological behaviour. So the question remains, as to why there are differences in the direction of the effects of these compounds in the present systems.

We are indebted to Mrs. *U. Häffelin* and Miss *M. Wecker* for their excellent technical assistance. We are also grateful to Dr. *A.-M. Fretzdorff* for carrying out the elemental analyses.

Literature

[1] Weitzel, G., Renner, R. & Guglielmi, H. (1971) *this J.* **352**, 1617 - 1630.
[2] Weitzel, G., Renner, R. & Guglielmi, H. (1972) *this J.* **353**, 535 - 539.
[3] Weitzel, G., Stock, W. & Guglielmi, H. (1972) *this J.* **353**, 1661 - 1670.
[4] Lockwood, D. H. & East, L. W. (1974) *J. Biol. Chem.* **249**, 7717 - 7722.
[5] Bihler, J. & Jeanrenaud, B. (1970) *Biochim. Biophys. Acta* **202**, 496 - 506.
[6] Rudinger, J., Poduska, K. & Zaoral, M. (1960) *Collect. Czech. Chem. Commun.* **25**, 2022 - 2028.
[7] Bello, J. (1955) *Biochim. Biophys. Acta* **18**, 448.
[8] Tesser, G. J. & Nispen, J. W. van (1971) *Synth. Comm.* **1**, 285 - 287.
[9] Eisele, K. & Schwegler, F. (1975) *Liebigs Ann. Chem.* in press.
[10] Boissonas, R. A., Guttmann, S., Huguenin, R., Jaquenoud, P. & Sandrin, E. (1958) *Helv. Chim. Acta* **41**, 1867 - 1882.
[11] Rodbell, M. (1964) *J. Biol. Chem.* **239**, 357 - 380.
[12] Kalbhen, D. A. & Koch, H. J. (1967) *Z. Klin. Chem. Klin. Biochem.* **5**, 299 - 309.
[13] Kurtz, C. A. (1949) *J. Biol. Chem.* **180**, 1253 - 1267.
[14] Ditschuneit, H., Rott, W. H. & Faulhaber, J. D. (1968) 2 nd. Int. Biguanidsymposion, 1967, Thieme-Verlag, Stuttgart.