Vasodilator effect of glucagon, glucagon-like peptide-1 and GLP-1 receptor agonists, and role of oxidative stress in their vasoactivity

Ph.D. thesis summary

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1. Introductuion

Glucagon is produced by the alpha cells of the Langerhans islets of the pancreas from its precursor, proglucagon, a 160-amino acid polypeptide (1). Proglucagon is also the precursore molecule for two other important peptide hormones, glucagon-like peptide-1 and 2 (GLP-1, GLP-2) produced in the L-cells of the small intestine (1). Glucagon antagonises the metabolic effects of insulin, while GLP-1, produced in response to food intake, enhances the secretion of insulin (1).

Apart from its blood glucose elevating effect, glucagon exhibits several extrahepatic effects (1). In the heart it exerts positive inotropic and chronotropic effects, in the gastrointestinal tract it acts as a smooth muscle relaxant, while it also reduces vascular resistance in several organs (1). Although the vasodilator effect of glucagon was formerly suggested, for instance in renal arteries of rabbits (2), however, there has been no direct evidence that glucagon may induce vasodilatation in certain vessels, and the mechanism of the vasodilatation has also not been clarified.

Taking advantage of its blood glucose lowering effects, the long acting mimetics of GLP-1 are widely used in the treatment of type 2 diabetic patients (3). Beside their antidiabetic effects, GLP-1 mimetics also have beneficial cardiovascular effects (4). On the other hand, it is not so widespread, that GLP-1 relaxes certain arteries, which could be an explanation for the blood pressure lowering effect of GLP-1 mimetics.

It has been demonstrated previously, that native GLP-1 causes dose-dependent relaxation of the thoracic aorta, pulmonary and femoral artery of rats (5,6,7). Nitric oxide (NO), cAMP and ATP-sensitive potassium channels were discovered to be mediating the vasodilatation (5,6), however, through a mechanism not thoroughly described.

The dose-dependent vasodilator effect of exendin-4 (exenatide) in the rat aorta has previously been demonstrated, although the mechanism is not precisely described (8).

Another GLP-1 mimetic, liraglutide induces nitric oxide production in vascular endothelial cells, however, there was no evidence for its direct vasodilator effect previously (9).

Oxidative stress processes play an important role in the development and progression of chronic diseases, for example type 2 diabetes (10).

As a result of oxidative stress, due to hydroxyl free radical, phenylalanine is transformed not only to the physiological amino acid para-tyrosine, but also into two other tyrosine isoforms, meta-tyrosine, and ortho-tyrosine. Para-tyrosine is formed in higher amount via an enzymatic reaction, while meta- and ortho tyrosine are only formed due to free radicals. The imbalance

2

between the physiological and pathological-tyrosine isomers might lead to the development of insulin-, acetylcholine- and erythropoietin-resistance (11,12).

Former data of our workgroup indicated that the accumulation of oxidized amino acids (meta- and ortho-tyrosine) due to oxidative stress, may play an important role in the impaired insulin-induced vasoactive properties of different arterial segments (13). There are evidences, that incorporation of these amino acids into cellular proteins may lead to certain hormonal resistances, which might be restored by supplementation with the physiological isoform, para-tyrosine.

2. Aims

2.1.   Study of the vasoactivity of GLP-1 and its mimetics

- We aimed to find direct evidence for the hypothesis that glucagon induces vasodilatation of the rat thoracic aorta *in vitro*.

- We also aimed to determine the mediators of the glucagon-induced vasodilatation.

- We wanted to compare the vasoactive potential of glucagon, GLP-1 and insulin.

- We aimed to demonstrate that exenatide and liraglutide induces vasodilatation in the rat thoracic aorta *in vitro*.

- We also aimed to prove that all three gasotransmitters, nitric oxide (NO), carbon monoxide (CO) and hydrogen sulphide (H_2S) are involved in the vasodilatation induced by exenatide and liraglutide.

- We wanted to clarify the mediators of the vasodilatation induced by exenatide and liraglutide.

2.2.   The role of oxidative stress in the vasodilatation induced by liraglutide and insulin

- We aimed to prove that the physiological amino acid, para-tyrosine is capable of restoring the hypercholesterolaemia, thus oxidative stress induced increased pathological tyrosine content of the vascular wall, thus attenuating the functional vascular damage.

3. Methods

3.1. Vasoreactivity experiments

The vasoactive effect of glucagon, insulin, GLP-1, exenatide and liraglutide was studied on isolated thoracic aortic rings of adult rats. We performed our experiments with the permission of the Hungarian Local Animal Experiment Committee. The vasoactive effect of liraglutide was also studied on rat femoral artery. Rats were anaesthetized with ether and decapitated, then the thoracic aorta and the femoral artery were isolated, and placed into ice-cold, oxygenated (95% O_2/5% CO_2) Krebs solution (119 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 11.1 mM glucose, 1.6 mM $CaCl_2*2H_2O$, pH 7.4). Two millimetre-long vessel segments were mounted in a wire myograph and after the normalization process; vessels were incubated with potential inhibitors of vasodilatation. Then, vessels were preconstricted with epinephrine (100 nM), and as the vessel segments had reached a contraction plateau, increasing dosages of vasodilator, -insulin, glucagon, GLP-1, exenatide or liraglutide- were administered to the myograph chambers. To investigate the mechanism of the vasodilatation caused by glucagon, we determined the role of the receptor for glucagon and the receptor for GLP-1. To investigate mediators involved in the vasodilatation evoked by glucagon, exenatide and liraglutide, we studied the effect of various inhibitors of the enzymes producing the three gasotransmitters- nitric oxide, carbon monoxide and hydrogen sulphide (L-NAME, Tin-Protoporphyrin, D-L-Propargylglycine), with inhibitors of reactive oxygen species formation (superoxide dismutase, catalase), NADPH oxidase (diphenlyeneiodonium chloride), prostaglandin synthesis (indomethacin), inhibitors of protein kinases (H89 hydrochloride, 1H-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one), inhibitors of potassium channels (glibenclamide, tetraethylammonium, XE991) and an inhibitor of the Na^+/Ca^{2+}-exchanger (SEA0400).

3.1.1. Statistical analysis

Myodaq 2.01 M610+ software was used for data acquisition and display. We expressed the rate of relaxation caused by insulin, GLP-1, glucagon, exenatide, and liraglutide as the percentage of the contraction evoked by epinephrine.

Statistical analysis was performed using SPSS Version 22.0 and GraphPad Prism 6.0. Statistical significance was calculated using repeated measures ANOVA with Bonferroni post hoc test. Values are shown as mean ±SE. A value of P less than 0.05 was considered to be significant.

3.2. Metabolic animal model

3.2.1. Animals

Experiments were performed with the permission of the Hungarian Local Animal Experiment Committee. We started our oxidative stress study with four weeks old Sprague-Dawly rats. The animals were divided into three groups. Rats in the control group were kept on a regular diet (n=10), rats in the cholesterol-fed group (n=10) received high-fat diet (70 cal% fat), while the third group of rats (n=10) received high-fat diet with para-tyrosine supplementation (1.76 mg/die) for 16 weeks.

On the day of the experiment, rats were anaesthetized with ether, then blood sample was collected via cardiac puncture and the thoracic aorta was removed.

3.2.2. Vasoreactivity experiments

The vascular response to insulin- and liraglutide was evaluated with a DMT multi myograph. Following the normalization process, vessel segments were preconstricted with epinephrine (100 nM) and then either increasing doses of insulin or liraglutide were applied to the chambers.

3.2.3. Investigation of metabolic parameters

Plasma total-cholesterol level was determined using a one-step enzymatic method with fluorescence spectrophotometry.
Plasma concentrations of insulin were measured using a Rat Ultrasensitive Insulin ELISA kit (Alpco), detected with a microplate spectrophotometer.

3.2.4. Measurement of tyrosine isoforms in the rat thoracic aorta

An overnight hydrolysis in hydrochloric acid was performed at 120°C on equal sized thoracic aortic segments, the precipitate was separated by centrifugation, and then it was filtered before analysis. Finally levels of para- and meta-tyrosine were determined using reverse phase-HPLC with fluorescence detection (λEX=275 nm; λEM=305 nm)

3.2.5. Statistical analysis

Statistical analysis was performed by using GraphPad Prism 6.0 and SPSS Version 22.0. Kolmogorov-Smirnov test was used as a test of normality. Intergroup analyses were performed using Student's t-test or ANOVA with Bonferroni post hoc test as appropriate, in case of normal distribution. Kruskal-Wallis test followed by Mann-Whitney U test was used

when distribution was not normal. Jonckheere-Terpstra test was used to assess tendency. Values are shown as mean±SE or median (interquartile range). Values of P less than 0.05 were considered significant.

4. Results

4.1. Vasoactive effect of glucagon and glucagon-like peptide mimetics

4.1.1. Glucagon

Glucagon dilates the rat thoracic aorta

Glucagon caused dose-dependent vasodilatation of the rat thoracic aorta, which was as effective, as the vasodilatation evoked by insulin. Glucagon and insulin proved to be more potent vasodilators in the rat thoracic aorta than native GLP-1(7-36).

Receptorial crosstalk between glucagon, GLP-1, glucagon receptor and GLP-1 receptor

The vasodilatation evoked by GLP-1 was partially mediated by the glucagon-receptor, while the glucagon-induced vasodilatation evoked mostly via the glucagon-receptor but also via the GLP-1 receptor, independent of the vascular endothelium.

Further mediators of the glucagon induced vasodilatation

Gasotransmitters, prostaglandins, the NADPH oxidase enzyme, free radicals, potassium channels and the Na^+/Ca^{2+}-exchanger are also involved in the vasodilatation induced by glucagon.

4.1.2. Exenatide

Exenatide induces vasodilatation via the GLP-1-receptor

Exenatide also causes dose-dependent vasodilatation of the rat thoracic aorta, which is – according to our findings- mediated by the GLP-1 receptor.

Mediators of the vasodilatation evoked by exenatide

Exenatide induces vasodilatation mainly via hydrogen sulphide (H_2S) but also via nitric oxide (NO) and carbon monoxide (CO). Prostaglandins and superoxide free radical also play a part in the relaxation. Inhibition of the soluble guanylyl cyclise (sGC) significantly diminished vasorelaxation. We found that ATP-sensitive-, voltage-gated- and calcium-activated large-conductance potassium channels are also involved in the vasodilatation, but seemingly the inhibition of the KCNQ-type voltage-gated potassium channels resulted in the most remarkable decrease in the rate of vasorelaxation. Inhibition of the Na^+/Ca^{2+}-exchanger abolished most of the vasodilatation.

4.1.3. Liraglutide

Vasoactive effect of liraglutide

According to our findings, liraglutide relaxes the rat thoracic aorta and femoral artery *in vitro*. The vasorelaxation evoked in a dose-dependent manner.

Mechanism of the liraglutide-induced vasodilatation

According to our findings, liraglutide activates endothelial cells and vascular smooth muscle cells leading to the production of NO, CO, H_2S, superoxide anion, and hydrogen peroxide. Increased production of such relaxing factors promotes the activation of protein kinase- A and -G, resulting in the activation of potassium channels (ATP-sensitive-, voltage-gated-, large-conductance-calcium activated), which profoundly contributes to the activation of the Na^+/Ca^{2+}-exchanger, thereby leading to calcium efflux and smooth muscle relaxation and vasorelaxation.

4.2. Effect of oxidative stress in cholesterol-fed rats

Metabolic parameters of cholesterol-fed rats and rats with para-tyrosine supplementation

In the oxidative stress study, plasma cholesterol level was significantly higher in the cholesterol-fed group, while the level of cholesterol in the cholesterol + para-tyrosine group did not differ significantly from that of the controls. Plasma level of insulin after glucose stimulation was decreased in the cholesterol-fed group, while that was not significantly different from the controls in the para-tyrosine supplemented group.

Change of the tyrosine-isomer concentration of the vascular wall as a result of cholesterol-feeding and para-tyrosine supplementation

Elevated vascular meta-tyrosine/para-tyrosine ratio, as a sign of oxidative-stress in cholesterol fed rats, could be avoided by para-tyrosine supplementation.

Vascular response to liraglutide- and insulin in cholesterol fed rats and in rats with para-tyrosine supplementation

Cholesterol feeding resulted in vascular insulin-and liraglutide resistance, which was restored by para-tyrosine supplementation.

5. Discussion

5.1. Mechanism of the glucagon induced vasodilatation

Glucagon causes dose-dependent vasorelaxation of the rat thoracic aorta *in vitro*, which evokes via the receptor for glucagon and the receptor for GLP-1.

Metabolic actions of glucagon evolve via the glucagon receptor (1), however, it has not been investigated whether its vasodilator effect is transmitted via the glucagon receptor. At the same time, the vasodilatation induced by GLP-1 partially evoked via the glucagon receptor, thus it is possible that a cross-talk of the glucagon- and GLP-1-induced vasodilatation exists.

As mentioned before, type 2 diabetes is commonly treated by analogues of native GLP-1 and dipeptidyl peptidase-4 inhibitors, inhibitors of the enzyme degrading incretin hormones (3). These drugs act via increasing the level of GLP-1, and are also known to decrease the level of glucagon (15). Like native GLP-1, its mimetics also cause vasodilatation (6). Speculatively, based on our novel findings, the drugs that increase the level of GLP-1, might also induce vasodilatation via the glucagon receptor. Moreover, glucagon and its receptors have been suggested to be potential targets for the treatment of type 2 diabetes and its complications (16).

The role of NO and prostaglandins in the glucagon-induced vasodilatation have previously been suggested (17), but we went further and revealed that all of the three gasotransmitters, nitric oxide, hydrogen sulphide and carbon monoxide, reactive oxygen species (ROS)-superoxide anion (O_2^{-}) and hydrogen peroxide (H_2O_2) are part of the vasodilatation induced by glucagon. Production of ROS is mediated by the NADPH oxidase, while these products may activate the sGC-cGMP-protein kinase G pathway, potassium channels and the Na^+/Ca^{2+}-exchanger, eventually leading to vasodilatation.

5.2. Mechanism of the vasodilatation induced by exenatide and liraglutide

Both GLP-1 and other related peptides are known to induce vasodilatation in central as well as peripheral vessels, however, the role of the glucagon-like peptide-1 receptor (GLP-1R) has not been clarified in the vasodilatation, since both GLP-1R-dependent and –independent vasodilator mechanisms of GLP-1 mimetics have been described (18).

In Glp1r^{-/-} mice, native GLP-1 reduced the ischemic damage after ischemia-reperfusion and also increased the production of cGMP, thereby leading to vasodilatation, and increased coronary flow (18). However, the same study reported GLP-1R dependent cardioprotective and glycaemic effects of native GLP-1 amide (18).

It has also been suggested that GLP-1 peptides induce vascular relaxation in a GLP-1R-independent manner, at least in the rat aorta, independently of its well-know metabolic actions (16).

In our experiments, exenatide caused dose-dependent relaxation of the rat thoracic aorta, which evoked via the GLP-1 receptor, while the role of GLP-1 receptor in the vasodilatation induced by liraglutide was not investigated. Liraglutide proved to be a more potent vasodilator in the femoral artery than in the thoracic aorta.

We found that exenatide and liraglutide caused partially endothelium-dependent vasodilatation, in contrast to a previous study, which found that the vasoactive effect of GLP-1 was independent of the endothelium (6). While previously only NO was shown to be involved in the vasodilatation induced by GLP-1 mimetics, we reveal, that all three gasotransmitters are involved in it (6). We showed for the first time, that prostaglandins and superoxide free radical, protein kinase-A and –G, potassium channels and the Na^+/Ca^{2+}-exchanger also play a part in the relaxation induced by GLP-1 mimetics. Inhibition of soluble guanylyl cyclase significantly diminished vasorelaxation. We found that ATP-sensitive-, voltage-gated- and calcium-activated large-conductance potassium channels are also involved in the vasodilatation, but that seemingly the inhibition of the KCNQ-type voltage-gated potassium channels resulted in the most remarkable decrease in the rate of vasorelaxation. Inhibition of the Na^+/Ca^{2+}-exchanger abolished most of the vasodilatation.

It is known that GLP-1 mimetics decrease both systolic and diastolic blood pressure, while we proved *in vitro* in our experiments that they might as well decrease central blood pressure. Our findings are also relevant in the clinical context, since central blood pressure is a good predictor of cardiovascular outcomes (19).

5.3. Effect of oxidative stress on the vasodilatation induced by liraglutide and insulin

The treatment of type 2 diabetes has improved in the past decades as the underlying pathomechanisms were recognised (20). The ominous octet is well known to describe the factors contributing to the development of glucose intolerance: insulin resistance of the pancreatic β-cells, muscle, liver and the brain; incretin resistance or deficiency; hyperglucagonaemia; increased glucose reabsorption and accelerated lipolysis (20). The identification of these factors resulted in a paradigm shift in the treatment, however, prevention of the disease still cannot be enough emphasized. With better understanding of the underlying molecular mechanisms, a more effective prevention would be available.

High-fat diet (HFD) induced obesity is the most widely studied model of type 2 diabetes (21). In our study, the rats did not develop obesity, but they had hypercholesterolaemia, and exhibited an important feature observed in T2DM, namely decreased glucose-stimulated insulin secretion. The cholesterol-induced decreased glucose-stimulated insulin secretion could be restored with the supplementation of the physiological amino acid, para-tyrosine. This supports our hypothesis, that para-tyrosine might be useful in the restoration of the oxidative stress-induced damaged vascular response to insulin.

It is well known, that the level of the oxidative stress marker ortho-tyrosine is elevated in type 2 diabetes, and the concentration of ortho-tyrosine was also reported to be increased in the aortic proteins of diabetic monkeys (22,23). The role of reactive oxygen species is also known in the development of the diabetes mellitus associated endothelial dysfunction (24).

The present study is a part of a series of experiments regarding the possible role of the pathological and physiological tyrosine isoforms. In our first study, higher ortho-tyrosine content was found in the central blood vessels of rats, while with the decrease of ortho-tyrosine concentration toward the peripheral vessels, insulin-induced vasorelaxation increased (25). In our second study, oral ortho-tyrosine treatment of rats for 4 weeks significantly increased vascular ortho-tyrosine content, and at the same time, impaired insulin-induced relaxation was demonstrated in isolated femoral arteries (13).

In our present study, elevated vascular meta-tyrosine content of the aortic wall, leading to vascular liraglutide-and insulin resistance in cholesterol fed rats, could be avoided by para-tyrosine supplementation. The clinical significance of our findings is that a physiological material is capable of restoring the oxidative stress induced functional vascular damage.

6. List of the Ph.D. theses

1. Glucagon induces dose-dependent vasodilatation via the glucagon- and the GLP-1-receptor *in vitro.*

2. Further mediators of the glucagon induced vasodilatation are the NADPH oxidase, free radicals, gasotransmitters, prostaglandins, PKA, sGC, potassium channels and the Na^+/Ca^{2+}-exchanger.

3. The vasodilatation induced by native GLP-1 is partially evoked via the glucagon-receptor.

4. GLP-1 mimetics exenatide and liraglutide induce dose-dependent vasorelaxation of the rat thoracic aorta *in vitro*, via H_2S but also via NO, CO,

$O_2^{-\bullet}$ and prostaglandins, and this effect can be mediated via the activation of PKA and PKG. Through the induction of these mediators, exenatide and liraglutide also influence the activity of potassium channels and the Na^+/Ca^{2+} -exchanger.

5. The relaxation evoked by liraglutide in the rat femoral artery is greater than that in the thoracic aorta.

6. Cholesterol feeding results in increased meta-tyrosine concentration in the wall of the rat thoracic aorta associated with decreased vascular response to liraglutide and insulin.

7. Supplementation with the physiological amino acid para-tyrosine restores hypercholesterolemia induced increased meta-tyrosine content of the vascular wall, thus attenuates functional vascular damage.

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8.1. List of publications not related to the thesis

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Mikolás E, Kun S, Laczy B, Molnár GA, **Sélley E**, Kőszegi T, Wittmann I. Incorporation of Ortho- and Meta-Tyrosine Into Cellular Proteins Leads to Erythropoietin-Resistance in an Erythroid Cell Line. *Kidney Blood Press Res* 2014 Apr 9;38(2-3):217-225.IF: 1.82

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Cumulative impact factor: 18.66

Number of independent citations: 14

8.2. List of presentations and abstracts

Kun Szilárd, Mikolás E., **<u>Sélley E</u>**., Wittmann I.:Az oxidált aminosav, orto-tirozin szerepe az erythropoetin-rezisztencia kialakulásában. *I. MATHINÉ Budapest, 21.03.2013.*

<u>Sélley E</u>, Kun Sz., Molnár G., Degrell P., Kürthy M., Kovács T., Wittmann I.: Para-tirozin szupplementáció javítja az inzulin- és a liraglutide-kiváltotta vazorelaxációt koleszterinnel etetett patkányokban. *II. MATHINÉ Budapest, 14.03.2014.*

Mikolás Esztella Zsóka,Kun Sz**.**, Laczy B., Molnár G.A., **<u>Sélley E</u>**., Kőszegi T., Wittmann I.: A cukorbetegekben tapasztalt korai anaemia lehetséges oka. *II. MATHINÉ Budapest, 14.03.2014.*

<u>Sélley E</u>, Molnár G.A., Kun Sz**.**, Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I. A liraglutid komplex vazoaktív hatása. Három gázmolekula jelátvivő szerepe. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) p. &. (2014)*

Kun Szilárd, **<u>Sélley E</u>**., Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I., Molnár G.A. Az exenatid a kén-hidrogén, a szén-monoxid és a nitrogén-monoxid termelődésének fokozása révén kelthet centrális vasodilatatiót. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) p. &. (2014)*

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Molnár Gergő Attila, **Sélley E.,** Kun Sz**.**, Degrell P., Kürthy M., Kovács T., Wittmann I. Para-tirozin szupplementáció javítja az inzulin- és a liraglutid kiváltotta vasorelaxatiót koleszterinnel etetett patkányokban. *MDT XXII. Kongresszusa Szeged, 26.04.2014.DIABETOLOGIA HUNGARICA 22:(S2) pp. 105-107. (2014)*

Mikolás Esztella,Kun Sz., Laczy B., Molnár G.A., **Sélley E.**, Kőszegi T., Wittmann I.: Az orto- és meta-tirozin beépülés in vitro erythropoetin-rezisztenciához vezet. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

Sélley E., Molnár G.A., Kun Sz**.**, Szijártó I.A., Laczy B., Kovács T., Fülöp F., Wittmann I.: A GLP-1 analógok vese artériára gyakorolt hatása. Gázmolekulák jelátvivő szerepe. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

Molnár Gergő Attila,Kun Sz., Mikolás E., **Sélley E.**, Csiky B., Wittmann I.: Az erythropoetin-rezisztencia és az oxidált aminosav, az orto-tirozin összefüggése. *MANET XXXI. Nagygyűlése Eger, 07.11.2014.*

E. Sélley, Sz. Kun, I.A. Szijártó, B. Laczy, T. Kovács, F. Fülöp, I. Wittmann, G.A. Molnár: Vasodilatation by lixisenatide in the rat thoracic aorta, mediated by an unknown receptor. Third International Symposium on Hypertension, Translational Medicine in Hypertension. *Osijek*, 28.11.2014.

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Resveratrol hatásának vizsgálata IgA nephropathiában. (Pilot vizsgálat)

Kovács Tibor**,** Brasnyó P., Molnár G.A., **Sélley E**., Kun Sz., Vas T., Laczy B., Fekete K., Kovács K., Mészáros G.L., Winkler G., Sümegi B., Wittmann I.*MANET XXXII. Nagygyűlése és Továbbképző Tanfolyama Siófok*, 12.11.2015.

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