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The poliovirus-induced shut-off of cellular protein synthesis persists in the presence of 3-methylquercetin, a flavonoid which blocks viral protein and RNA synthesis

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Summary

In poliovirus-infected cells, the viral protein and RNA synthesis were severely reduced, provided 3-methylquercetin was present between 1 and 2 h post-infection. Under these conditions, the virally induced host shut-off remained in effect. On the other hand, in uninfected HeLa cells, protein and RNA synthesis was inhibited only slightly by 3-methylquercetin. The inhibition of poliovirus cytopathogenicity in Vero cells by 3-methylquercetin exhibited a similar time dependence.

3-methylquercetin; poliovirus; shut-off

Introduction

In an antiviral screening program of higher plants, crude extracts of the African plant *Euphorbia grantii* Oliver were found to exhibit a pronounced activity against several picornaviruses. The constituents responsible for the antiviral effect were isolated and identified as four related 3-methoxyflavones [6]. One of these flavones, 3,3′,7-trimethylquercetin, previously isolated from a Chinese medicinal herb, was found to inhibit the replication of several picornaviruses. In HeLa cells in-

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fected with rhinovirus type 2 or coxsackie B1 virus, this compound inhibited the formation of progeny virus when added between 2 and 4 h post infection. RNA synthesis of both viruses was completely inhibited when the compound was added 1 h post infection. On the other hand, viral RNA polymerase activity isolated from infected cells was not inhibited. In mock infected cells, no effect on cellular RNA synthesis was observed [3].

4′,5-Diacetyl-3,3′,7-trimethylquercetin protected mice against lethal infection by coxsackievirus when given orally [3].

The four related 3-methoxyflavones isolated by Van Hoof et al. [6] were tested in poliovirus type 1 infected Vero cells. As 3-methylquercetin (3-MQ) showed the highest antiviral activity and protected mice against coxsackie B4 infection, we used this compound in our experiments. As will be shown, 3-MQ inhibited the viral protein and RNA synthesis in HeLa cells only if present at certain times post infection.The inhibition of poliovirus induced cytopathy in Vero cells was also time dependent.

The virally induced shut-off of HeLa cell protein synthesis remained in effect in the presence of 3-MQ, even though the viral RNA and protein synthesis was reduced to an undetectable level. The possible implications of this observation are discussed.

Materials and Methods

Labeling and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of poliovirus proteins

HeLa cell suspension cultures (10^7 cells/ml) were infected with type 1 poliovirus (Mahoney strain) at a multiplicity of 100 plaque forming units per cell in methionine-free medium, and 5% calf serum was added after 30 min. The cells received 3-MQ at various times. At 3 h post-infection (p.i.), 5 μCi/ml [^{35}S]methionine was added. Incubation was continued and samples were withdrawn in order to determine the acid precipitable radioactivity. At 3.5 h p.i., the cells were washed with ice-cold medium containing an excess of unlabelled methionine, collected by low speed centrifugation, and resuspended in 90 μl of distilled water. 10 μl of a solution of 10% SDS and 10% β-mercaptoethanol in 0.5 M Tris-HCl, pH 6.9, was added. The samples were then boiled for 5 min, mixed with 20 μl loading buffer (containing 80% sucrose; 1% β-mercaptoethanol; 0.1% bromophenol blue and 0.05 M Tris-HCl, pH 6.9) and loaded on a 12.5% polyacrylamide slab gel. Electrophoresis was performed at 1.3 $V \cdot cm^{-1}$ until the dye reached the bottom of the gel. Gels were stained with Coomassie Brilliant Blue R 250 and soaked in EN^{3}HANCE™ (NEN). Fluorograms of the dried gels were made using Kodak X-Omat S film.

[^{3}H]Uridine incorporation in viral RNA

To HeLa cell suspension cultures (10^7 cells/ml), actinomycin D (5 μg/ml) was added 30 min before infection. The cells were infected with 100 plaque forming

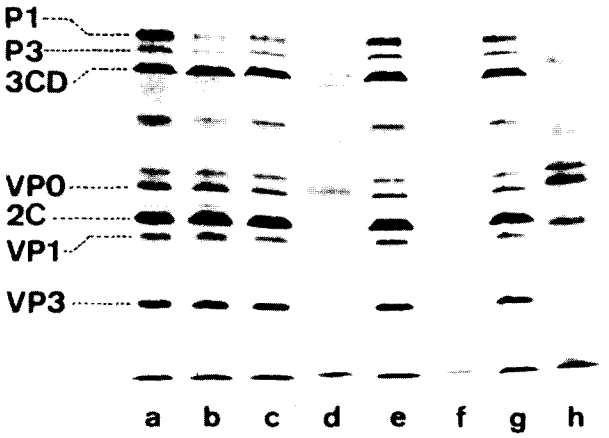

Fig. 1. Effect of 3-MQ on the synthesis of viral proteins in infected HeLa cells. Cultures a to g were infected with poliovirus, and h was a mock infected control. 3-MQ was added at 30 min p.i.; All cultures received [^{35}S]methionine at 3 h p.i.; The cells were collected 30 minutes later, and the labelled proteins were analysed by SDS-PAGE. Lanes b,d and f: 3, 10 and 30 μg/ml 3-MQ, respectively; Lanes a, c, e, and g: no 3-MQ; Lane h: mock infected cells, no 3-MQ.

units per cell, and [^{3}H]uridine (5 μCi/ml) was added 30 min later. 3-MQ was added 35 min p.i. in various concentrations. The radioactivity incorporated in cold TCA precipitable material was measured at various times p.i.

Results

Effect of 3-MQ on protein and RNA synthesis in poliovirus infected HeLa cells

HeLa cell suspensions were infected and 3-MQ in various concentrations was added at 30 min p.i. [^{35}S]methionine was given from 3 to 3.5 h p.i., and the pattern of labelled proteins was analysed by SDS-PAGE (Fig. 1). The labelling of the viral proteins was normal with 3 μg/ml 3-MQ (lane b), and was severely suppressed by 10 (lane d) or 30 μg/ml (lane f). The concentration of 10 μg/ml 3-MQ was used in further experiments. This concentration is also known to inhibit viral protein synthesis in poliovirus infected Vero cells [6].

Simultaneously infected HeLa cell cultures received 10 μg/ml 3-MQ at times ranging from 5 min before to 3 h after infection. In each case, the rate of protein synthesis was measured over a 32 min period starting at 3 h p.i. (Table 1). The pattern of proteins labelled during this period was examined using SDS-PAGE (Fig. 2).

In infected cells, the well-known phenomenon [1,2] of shutoff of cellular protein synthesis was observed. The incorporation rate was reduced to 22% of that in uninfected cells (Table 1, last line), and all the label went into viral proteins only (Fig. 2, lane j). When 3-MQ was added just before infection (lane d) or at 45 min

TABLE 1

Effect of 3-MQ added at various times p.i. on the rate of protein synthesis in infected and mock infected cells

Time of addition of 3-MQ (min p.i.)	Protein labelling patterns		Incorporation rate (% of control)
	Lane in Fig. 2 showing pattern	Interpretation (C = cellular, V = viral)	
Mock infected			
−5	b	C	77
180	c	C	98
no 3-MQ	a	C	100
Infected			
−5	d	C	19
45	e	C	12
90	f	C+V	5
120	g	V	11
150	h	V	19
180	i	V	23
no 3-MQ	j	V	22

Ten HeLa cell suspension cultures were either mock infected (a–c), or infected with poliovirus (d–j), as described in Materials and Methods. Cultures b to i received 3-MQ (10 μg/ml) at the indicated times. [^{35}S]Methionine was added to all cultures at 3 h p.i. Samples were withdrawn after 2, 4, 8, 16 and 32 min to measure the hot TCA precipitable radioactivity. Incorporation rates were calculated from the slopes, and expressed as percent of that in the mock infected culture without 3-MQ. The cells were collected at 3 h 32 p.i., and the labelled proteins analysed by SDS-PAGE (see Fig. 2).

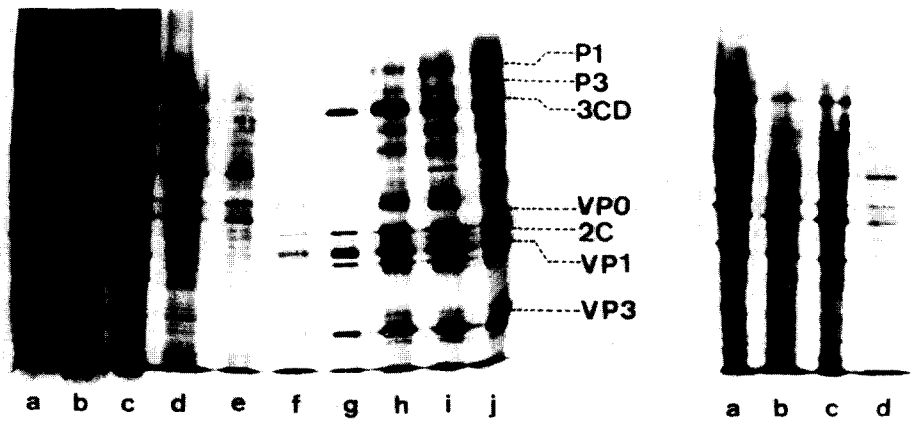

Fig. 2. Effect of 3-MQ added at various times p.i. on the pattern of protein labelling. Labelled proteins were prepared as described in Table 1 and analysed by SDS-PAGE. The fluorogram depicted in its entirety (lanes a–j) was made after a 12-day exposure of the dried gel. The gel was reexposed for 4 days. Lanes a–d of this second fluorogram are shown to the right, for a clearer view of the cellular protein labelling pattern. Lanes a–j: see Table 1.

p.i. (lane e), viral protein synthesis was completely suppressed, but the rate of labelling of the cellular proteins remained low (19 and 12% of the uninfected control, respectively; Table 1, lines d and e). The lowest rate of incorporation (5% of control) was obtained when 3-MQ was added at 1.5 h p.i., and the residual incorporation went into viral as well as cellular proteins (Fig. 2, lane f). When 3-MQ was added at 2 h p.i. or later, the labelling of cellular proteins was entirely suppressed and the rate of labelling of the viral proteins gradually approached that noted in the absence of 3-MQ (Table 1, lines g–j). In conclusion, even though no viral protein synthesis was detectable when 3-MQ was added early in the infectious cycle, cellular protein synthesis in infected cells remained severely depressed (e.g., to 12% of control when 3-MQ was added at 45 min p.i., Table 1, line e). This inhibition was mainly due to viral host shutoff and cannot be explained as direct inhibition by 3-MQ, as this compound in uninfected cells caused only 23% reduction in the incorporation rate when added 3 h in advance of the isotope, and no reduction when added simultaneously.

To complete this study of the effect of 3-MQ on viral protein synthesis, the compound was added to replicate cultures near the time of infection, and removed at various times thereafter. A viral pattern was obtained when 3-MQ was removed at 1 h p.i. (Fig. 3, lane b). Some cellular bands were present when the compound was allowed to act for 1.5 h p.i. or longer (Fig. 3, lanes c-d-e).

The combined results presented in Figs. 2 and 3 show that the time bracket in which 3-MQ must act to reduce the labelling of viral proteins lies between 1 and 2 h p.i.

To study the influence of 3-MQ on viral RNA synthesis, [^{3}H]uridine incorporation was measured in infected and mock infected cells treated with actinomycin

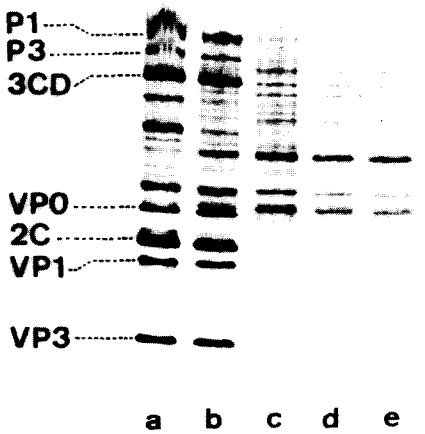

Fig. 3. Viral protein synthesis: effect of 3-MQ added before poliovirus infection and removed at different times thereafter. HeLa cell cultures a to e were infected and labelled as detailed in Materials and Methods. Cultures b to e received 3-MQ (10 μg/ml) 5 min before infection. The compound was removed at 1, 1.5, 2, or 2.5 hr p.i. (lanes b, c, d, and e, respectively). Lane a: no 3-MQ.

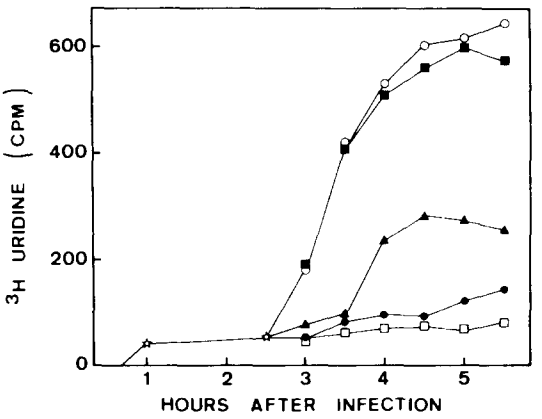

Fig. 4. Influence of 3-MQ on the viral RNA synthesis. Infected or mock infected HeLa cells pretreated with actinomycin D received [^{3}H]uridine at 30 min p.i. All the cultures except one received 3-MQ at 35 min p.i. Samples were taken at times shown to measure the cold acid precipitable radioactivity. ○—○, infected cells, no 3-MQ; ■—■, infected cells, 1 μg/ml 3-MQ; ▲—▲, infected cells, 3 μg/ml 3-MQ; ●—●, infected cells, 10 μg/ml 3-MQ; □—□, mock infected cells, no 3-MQ; ☆—☆, any of the foregoing.

D. As there was no measurable [^{3}H]uridine incorporation in mock infected cells, the RNA synthesis in infected cells (Fig. 4) was regarded as viral in agreement with previous findings [7]. When 3-MQ was added at 35 min p.i., the viral RNA synthesis was severely reduced by 10 μg/ml, but remained unaffected by 1 μg/ml. On the other hand, no inhibition of RNA synthesis by 3-MQ (10 μg/ml) was observed in mock infected cells in the absence of actinomycin D (results not shown). Thus, the dose-response curves for the inhibition of viral RNA (Fig. 4) and protein (Fig. 1) synthesis were roughly similar.

Influence of 3-MQ on the poliovirus induced cytopathic effect in Vero cells

It has been shown that 3-MQ prevented the development of cytopathy in poliovirus infected Vero cells [6]. It was therefore of interest to find out whether the protective effect was also dependent on the time of addition and removal of the compound. Replicate Vero monolayer cultures were infected with poliovirus. In the control monolayer which received no 3-MQ, all cells were rounded or disrupted after 48 h. The other monolayers received 10 μg/ml 3-MQ at 30-min intervals from 0.5 hr to 4 hr p.i. The results are given in Table 2, Expt. 1. No cytopathy developed when 3-MQ was given at 2 h p.i. or earlier. In contrast, the monolayers to which 3-MQ was given at 2.5 h p.i., or later, developed cytopathy. This result shows that 3-MQ must be present before 2.5 h p.i. to prevent cytopathy.

The lower time limit was located by adding 3-MQ at the time of infection, and removing it at various times thereafter (Table 2, Expt. 2). There was no protection when 3-MQ was removed as early as 0.5 or 1 h p.i. On the other hand, the cells were protected if the compound was allowed to act for 1.5 h p.i. or longer. It is concluded that the time during which 3-MQ exerted its protective action on infected Vero cells was comprised between 1 and 2.5 h p.i.

TABLE 2

Time limits for the protective action of 3-MQ against the development of poliovirus cytopathy in Vero cells

Time of 3-MQ addition (h p.i.):	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
Expt. 1: cytopathy after 48 h	–	–	–	–	+	+	+	+
Expt. 2: cytopathy after 48 h	+	+	–	–	–	–	–	–

+, extensive cytopathy comparable to the infected control without 3-MQ.
–, the monolayers remained indistinguishable from the uninfected control.
Confluent Vero cell monolayers were infected with 10 PFU/cell of type 1 poliovirus. After 48 h the monolayers were examined microscopically for cytopathy.
In Expt. 1, 10 μg/ml 3-MQ was added at the times shown, and not removed.
In Expt. 2, 10 μg/ml 3-MQ was added at the time of infection. At the times mentioned, the monolayers were washed and further incubated in medium without 3-MQ.

Discussion

The present studies show that the anti-picornavirus flavone 3-methylquercetin inhibited the viral protein and RNA synthesis in poliovirus infected HeLa cells. This effect depended on the presence of 3-MQ between 1 and 2 h p.i., suggesting that an early stage of the viral replication cycle was selectively inhibited. The protection of Vero cells by 3-MQ against virally induced cytopathy exhibited a similar time dependence.

Interestingly, the poliovirus induced shut-off of cellular protein synthesis persisted, even when the 3-MQ concentration was sufficient to completely inhibit viral RNA and protein synthesis. This suggests that the viral factor which caused the shut-off phenomenon did not depend on *de novo* protein synthesis. The persistence of poliovirus induced host shut-off under conditions preventing virus synthesis was also reported by Holland [2] and Bablanian [1]. A possible contribution of structural proteins to the shut-off is suggested by the observation that mutants with altered structural proteins were defective in the repression of host protein synthesis [5], and by the inhibitory effects of intact poliovirus particles on protein synthesis in reticulocyte lysates [4]. The possibility that host shut-off is induced by capsid proteins is being investigated.

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